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(54) **CHOLESTERINOXIDASE AUS BREVIBACTERIUM STEROLICUM**
CHOLESTEROL-OXIDASE FROM BREVIBACTERIUM STEROLICUM
CHOLESTEROL-OXYDASE DU BREVIBACTERIUM STEROLICUM

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- GENE. Bd. 103 , 1991 , AMSTERDAM NL Seiten 93 - 96 T. OHTA ET AL 'Sequence of gene choB encoding cholesterol oxidase of Brevibacterium sterolicum: comparison with choA of Streptomyces sp. SA-COO' In der Anmeldung erwähnt
- BIOSCIENCE, BIOTECHNOLOGY, AND BIOCHEMISTRY Bd. 56, Nr. 11 , November 1992 Seiten 1786 - 1791 T. OHTA ET AL 'Hyperexpression and analysis of choB encoding cholesterol oxidase of Brevibacterium sterolicum in Escherichia coli and Streptomyces lividans' In der Anmeldung erwähnt

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Beschreibung

[0001] Die Erfindung betrifft eine Cholesterinoxidase aus *Brevibacterium sterolicum*, ein Verfahren zur Herstellung einer rekombinanten Cholesterinoxidase aus *Brevibacterium sterolicum*, eine für dieses Verfahren geeignete DNA-Sequenz, welche eine zytoplasmatische Expression der rekombinanten Cholesterinoxidase im Wirtsbakterium bewirkt, sowie die so erhältliche rekombinante Cholesterinoxidase.

[0002] Für die enzymatische Bestimmung von Cholesterin ist die Cholesterinoxidase von großer Bedeutung. Sie katalysiert die Oxidation von Cholesterin zu Cholesten-3-on und H_2O_2 . Cholesterinoxidase aus verschiedenen Organismen wie *Pseudomonas*, *Mycobacterium*, *Nocardia*, *Arthrobacter* und *Brevibacterium* sind bereits beschrieben worden (T. Uwajima et al., Agr. Biol. Chem. 37 (1973), 2345 - 2350). Alle diese bekannten Cholesterinoxidasen sind sezernierte Proteine. Das Bodenbakterium *Brevibacterium sterolicum* KY 3643 (ATCC 21387) zeigt eine besonders hohe Aktivität der Cholesterinoxidase. Aus diesem Bakterium sind drei Isoenzyme der Cholesterinoxidase bekannt, die sich in ihrem isoelektrischen Punkt, der Substratspezifität gegenüber verschiedenen Steroiden, der Affinität gegenüber Cholesterin im pH-Optimum und der DNA bzw. Aminosäuresequenz unterscheiden (EP-A 0 452 112 und EP-A 560 983). Die Cholesterinoxidase I aus *Brevibacterium sterolicum* zeigt eine geringe Affinität zu Cholesterin (K_M $1,1 \times 10^{-3}$ mol/l) und ist aus *Brevibacterium sterolicum* nur in geringer Ausbeute erhältlich. Die Expression einer kompletten für die Cholesterinoxidase I kodierenden DNA in *E. coli* wurde bereits versucht, ist jedoch bislang nicht gelungen (K. Fujishiro et al., Biochem. Biophys. Res. Com. 172 (1990), 721 - 727, T. Ohta et al., Gene 103 (1991), 93 - 96). Auch die Expression spezieller Deletionsmutanten der für die Cholesterinoxidase I kodierenden DNA, welche mit Teilen des lac z Gens fusioniert wurden, führte zu keiner befriedigenden Expression in *E. coli* (T. Ohta et al., Biosci. Biotech. Biochem. 56 (1992), 1786 - 1791). In der EP-A 0 452 112 wird die Klonierung und Expression von weiteren Cholesterinoxidasen aus *Brevibacterium sterolicum* beschrieben. Die Expression dieser DNAs führt jedoch ebenfalls nicht zu einer ausreichenden Menge an aktiver Cholesterinoxidase.

[0003] Aufgabe der Erfindung war es, eine Cholesterinoxidase mit hoher Affinität zu Cholesterin in großen Mengen und in aktiver Form zur Verfügung zu stellen.

[0004] Diese Aufgabe wird gelöst durch eine Cholesterinoxidase, welche die in SEQ ID NO 2 gezeigte Aminosäuresequenz aufweist. Diese Cholesterinoxidase ist aus *Brevibacterium sterolicum* erhältlich oder auch rekombinant herstellbar.

[0005] Es hat sich überraschenderweise gezeigt, daß eine derartige Cholesterinoxidase rekombinant in großer Menge und in aktiver Form hergestellt werden kann. Diese Cholesterinoxidase weist ein Molekulargewicht von 60 kD, einen isoelektrischen Punkt von ca. 5,5 (jeweils gemessen im Phast-System, Pharmacia-LKB) sowie einen K_M -Wert für Cholesterin von 1×10^{-4} mol/l (in 0,5 mol/l Kaliumphosphatpuffer pH 7,5 bei 25°C) auf und ist in einem pH-Bereich von 5,5 bis 8,0 wirksam.

[0006] Es hat sich gezeigt, daß diese Cholesterinoxidase in großer Menge in aktiver Form erhalten werden kann, wenn für eine heterologe Expression eine DNA verwendet wird, welche für ein Peptid mit Cholesterinoxidase-Aktivität kodiert mit der in SEQ ID NO 1 gezeigten DNA-Sequenz oder der dazu komplementären DNA-Sequenz.

[0007] Vorzugsweise wird eine DNA verwendet, welche die in SEQ ID NO 1 gezeigte Sequenz aufweist. In dem Fachmann geläufiger Weise können jedoch degenerierte Codons durch andere Codons, welche für die gleiche Aminosäure kodieren, ersetzt werden. Zusätzlich soll die verwendete DNA eine der in SEQ ID NO 3, 4 und/oder 5 gezeigten DNA-Sequenzen aufweisen und für ein Peptid mit Cholesterinoxidase-Aktivität kodieren. Unter einem Peptid mit Cholesterinoxidase-Aktivität ist ein solches Peptid zu verstehen, welches die Oxidation von Cholesterin (5-Cholesten-3- β -ol) zu 4-Cholesten-3-on und H_2O_2 katalysiert.

[0008] Ein weiterer Gegenstand der Erfindung ist daher eine DNA, welche für ein Peptid mit Cholesterinoxidase-Aktivität kodiert mit der in SEQ ID NO 1 gezeigten DNA-Sequenz oder der dazu komplementären DNA-Sequenz.

[0009] Mit einer solchen DNA kann eine mindestens 10fach höhere Aktivität der rekombinant hergestellten Cholesterinoxidase im Rohextrakt erhalten werden als mit den bislang beschriebenen Verfahren und Cholesterinoxidasen.

[0010] Ein weiterer Gegenstand der Erfindung ist ein Verfahren zur Herstellung einer rekombinanten Cholesterinoxidase durch Transformation einer geeigneten Wirtszelle mit einer erfindungsgemäßen DNA, welche in einem geeigneten Expressionssystem vorliegt, Kultivierung der transformierten Wirtszellen und Isolierung der gebildeten Cholesterinoxidase aus dem Zytoplasma der transformierten Zellen.

[0011] Mit diesem Verfahren ist es überraschenderweise möglich, eine rekombinante Cholesterinoxidase in großer Menge und aktiver Form aus dem Zytoplasma der transformierten Wirtszelle zu erhalten. Dabei kann die verwendete DNA am 5'-Ende eine zusätzliche Nukleotidsequenz enthalten, die ein Translations-Startcodon, jedoch kein Stopcodon aufweist, wobei diese zusätzliche Nukleotidsequenz nicht zu einer Leserasterverschiebung führt und keine für die Sekretion des gebildeten rekombinanten Enzyms funktionell aktive Signalsequenz darstellt. Die Länge dieser Nukleotidsequenz beträgt etwa 3 bis 90 Basenpaare.

[0012] Vorzugsweise weist die zusätzliche Nukleotidsequenz eine der in den Sequenzprotokollen 6, 8, 10, 12, 14 und 16 gezeigten Sequenzen anstelle der nativen Signalsequenz auf.

[0013] Ein bevorzugter Gegenstand der Erfindung ist daher ein Verfahren zur Herstellung einer rekombinanten Cholesterinoxidase, wobei eine erfindungsgemäße DNA verwendet wird, welche am 5'-Ende eine der in SEQ ID NO 6, 8, 10, 12, 14 oder 16 gezeigten Sequenzen aufweist.

[0014] Die Transformation der für die rekombinante Herstellung verwendeten Wirtszellen erfolgt nach bekannten Verfahren (siehe z.B. Sambrook, Fritsch und Maniatis, "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989). Die transformierten Wirtszellen werden dann unter Bedingungen kultiviert, die eine Expression des Cholesterinoxidase-Gens erlauben. Je nach dem verwendeten Expressionsvektor ist hierfür in bekannter Weise gegebenenfalls die Zugabe eines Induktors (z.B. Lactose oder Isopropyl-β-D-thiogalactopyranosid (IPTG)) zum Kulturmedium, eine Temperaturerhöhung oder eine limitierte Glucosezufuhr zweckmäßig. Die Isolierung der rekombinanten Cholesterinoxidase aus dem Zytoplasma der transformierten Zellen erfolgt dann nach bekannten Verfahren.

[0015] Mit diesem Verfahren ist es möglich, die erfindungsgemäße Cholesterinoxidase als rekombinantes Enzym in einer Ausbeute von 8 - 20 U/ml zu erhalten. Die Expression des vollständigen Cholesterinoxidase-Gens, welches die Signalsequenz enthält, ergibt dagegen lediglich eine Ausbeute von unter 0,1 U/ml.

[0016] Ein bevorzugter Gegenstand der Erfindung ist eine erfindungsgemäße, für ein Peptid mit Cholesterinoxidase-Aktivität kodierende DNA, welche am 5'-Ende eine der in SEQ ID NO 6, 8, 10, 12, 14 und 16 gezeigten Sequenzen aufweist. Besonders bevorzugt sind die in den Sequenzprotokollen 18, 20, 22, 24, 26 und 29 gezeigten Sequenzen. Vorzugsweise liegen diese erfindungsgemäßen DNA-Sequenzen in einem Expressionsvektor kloniert vor. Mit Hilfe dieser DNA kann die erfindungsgemäße Cholesterinoxidase in beliebigen Mengen in den für die rekombinante Herstellung von Proteinen üblicherweise verwendeten Bakterien gewonnen werden. Vorzugsweise erfolgt die Expression in *E. coli*.

[0017] Ein weiterer Gegenstand der Erfindung ist daher eine rekombinante Cholesterinoxidase, welche von einer erfindungsgemäßen DNA kodiert wird und am N-terminalen Ende eine der in SEQ ID NO 7, 9, 11, 13, 15 oder 17 gezeigten Aminosäuresequenzen aufweist.

[0018] Diese rekombinante Cholesterinoxidase ist für einen enzymatischen Test zur Bestimmung von Cholesterin ebenso geeignet wie die übrigen aus dem Stand der Technik bekannten Cholesterinoxidasen. Falls erforderlich können in dem Fachmann geläufiger Weise durch in-vitro-Mutagenese zwischen diesen N-terminalen Sequenzen und der Aminosäuresequenz der reifen Cholesterin-oxidase Erkennungssequenzen für spezifische Proteasen wie z.B. der IgA-Protease, der Enterokinase oder des Faktors Xa integriert werden, so daß auch nach der zytoplasmatischen Expression der um diese N-terminalen Sequenzen verlängerten Cholesterinoxidase eine Abspaltung solcher anfusionierter N-terminaler Sequenzen möglich ist.

[0019] Ein bevorzugter Gegenstand der Erfindung ist eine rekombinante Cholesterinoxidase, welche die in SEQ ID NO 21, 23, 25, 27 oder 29 gezeigte Aminosäuresequenz aufweist, sowie die Verwendung einer solchen rekombinanten Cholesterinoxidase in einem enzymatischen Test zum Nachweis von Cholesterin. Dabei wird vorzugsweise das in der Cholesterinoxidasereaktion gebildete H₂O₂ in einer nachgeschalteten Indikatorreaktion als Maß für das in der Probe vorhandene Cholesterin bestimmt.

[0020] Die in den Beispielen genannten Plasmide pUC-Chol-B2-BB (DSM 8274), pmgl-SphI (DSM 8272) und pfl-20AT1-SD (DSM 8273) wurden am 05.05.1993 bei der Deutschen Sammlung für Zellkulturen und Mikroorganismen GmbH, Mascheroder Weg 1b, D - 3300 Braunschweig hinterlegt.

[0021] Die Anmeldung wird durch die folgenden Beispiele in Verbindungen mit den Sequenzprotokollen und Figuren näher erläutert.

SEQ ID NO 1 zeigt die Nukleinsäuresequenz der erfindungsgemäßen Cholesterinoxidase.

SEQ ID NO 2 zeigt die Aminosäuresequenz der erfindungsgemäßen Cholesterinoxidase.

SEQ ID NO 3 - 5 zeigen Nukleotidsequenzen aus erfindungsgemäßen, für ein Peptid mit Cholesterinoxidase-Aktivität kodierenden DNA's.

SEQ ID NO 6 - 17 zeigen die N-terminalen Sequenzen der erfindungsgemäßen rekombinanten Cholesterinoxidasene (SEQ ID NO 6, 8, 10, 12, 14 und 16) bzw. der dazugehörigen N-terminalen Aminosäuresequenzen (SEQ ID NO 7, 9, 11, 13, 15 und 17).

SEQ ID NO 18 - 29 zeigen die Nukleinsäuresequenzen und dazugehörigen Aminosäuresequenzen von erfindungsgemäßen rekombinanten Cholesterinoxidasen.

[0022] Dabei bedeuten:

Signalsequenz	vollständige Sequenz	Konstrukt
SEQ ID NO 6-7	SEQ ID NO 18-19	plac-Chol-cyt
SEQ ID NO 8-9	SEQ ID NO 20-21	ppfl-Chol-cyt
SEQ ID NO 10-11	SEQ ID NO 22-23	ppfl-MSN3H-Chol-cyt
SEQ ID NO 12-13	SEQ ID NO 24-25	ppfl-MSN4H-Chol-cyt
SEQ ID NO 14-15	SEQ ID NO 26-27	ppfl-MSN4R2K-Chol-cyt
SEQ ID NO 16-17	SEQ ID NO 28-29	ppfl-MVM3H-Chol-cyt

SEQ ID NO 30 - 33 zeigen vier Oligonukleotide für die Amplifikation eines Fragments des erfindungsgemäßen Cholesterinoxidase-Gens.

SEQ ID NO 34 zeigt die Sequenz eines Adapteroligonukleotids für die in vitro-Mutagenese des Cholesterinoxidase-Gens gemäß Beispiel 5.

Fig. 1 zeigt das Plasmid pUC-Chol-B2-BB.

Fig. 2 zeigt das Plasmid plac-Chol-cyt.

Fig. 3 zeigt das Plasmid ppfl-Chol-cyt.

Fig. 4 zeigt das Plasmid ppfl-MSN3H-Chol-cyt.

Beispiel 1

Klonierung des Gens für Cholesterinoxidase aus *Brevibacterium sterolicum*

[0023] *Brevibacterium sterolicum* (BMTU 2407) wird in 500 ml "nutrient broth" (Difco) 20 h bei 30°C angezüchtet. Die Zellen werden durch Zentrifugation geerntet. Die so gewonnene Zellmasse wird in 20 mmol/l Tris/HCl pH 8,0 zu 0,4 g Zell-Naßgewicht/ml resuspendiert. 2,5 ml dieser Suspension werden mit 5 ml 24 % (w/v) Polyethylenglycol 6000, 2,5 ml 20 mmol/l Tris/HCl pH 8,0 und 10 mg Lysozym versetzt und 14 h bei 4°C inkubiert. Dann erfolgt die Lyse der Zellen durch Zugabe von 1 ml 20 % (w/v) SDS und 2 mg Protease K und Inkubation für 1 h bei 37°C. Diese Lösung wird mit dem gleichen Volumen 20 mmol/l Tris/HCl pH 8,0 versetzt und dann pro ml 1 g CsCl sowie 0,8 mg Ethidiumbromid zugegeben. Diese Lösung wird durch Ultrazentrifugation 24 h bei 40.000 Upm in einem TV850 Vertikal-Rotor (DuPont) aufgetrennt. Die DNA-Bande wird dann mit einer Injektionsspritze abgezogen. Die Entfernung des Ethidiumbromids und Ethanol-Fällung der DNA erfolgt wie bei Sambrook et al., Molecular Cloning, A Laboratory Manual (1989) beschrieben.

[0024] 7 µg der so gewonnenen DNA werden partiell mit der Restriktionsendonuklease NlaIII (New England Biolab) geschnitten, auf einem 0,8 % Agarosegel elektrophoretisch aufgetrennt und ein Größenbereich von ca. 2 - 12 kb ausgeschnitten. Die DNA-Fragmente werden aus dem Gel isoliert, mit SphI geschnitten und anschließend in einen mit alkalischer Phosphatase aus Kälberdarm behandelten Plasmidvektor pUC19 ligiert. Dieser Ligationsansatz wird in kompetente *E. coli* K12 XL1-blue (Stratagene, Katalog-Nr. 200268) transformiert. Die transformierten Zellen werden auf Agarplatten mit LB-Medium, das 100 µg/ml Ampicillin enthält, ausplattiert und über Nacht bei 37°C inkubiert. Die hochgewachsenen Kolonien werden auf Nitrocellulosefilter (Schleicher und Schüll) übertragen, durch Behandlung mit Toluol/Chloroform-Dampf lysiert und die Filter mit der Kolonieseite auf Indikatorplatten (s.u.) übertragen. Auf diesen Indikatorplatten erfolgt der Nachweis auf eine Cholesterinoxidase-Aktivität durch 15- bis 30-minütige Inkubation bei Raumtemperatur.

[0025] Klone, die eine Farbreaktion zeigen, werden ausgewählt und isoliert. Zur Kontrolle werden diese *E. coli*-Klone auf einer Agarplatte mit LB-Medium, das 100 µg/ml Ampicillin enthält, ausgestrichen, über Nacht bei 37°C inkubiert, die angewachsenen Kolonien zur Verifizierung nochmals auf zwei verschiedene Nitrocellulosefilter transferiert und wie oben beschrieben mit Toluol/Chloroformdampf aufgeschlossen. Ein Filter wird wieder auf eine der oben beschriebenen Indikatorplatten aufgelegt, der andere Filter auf eine Indikatorplatte ohne Cholesterin. Eine positive Farbreaktion zeigt sich nur auf den kompletten Indikatorplatten mit dem Substrat Cholesterin. Damit wird nachgewiesen, daß die durch den entsprechenden *E. coli*-Klon hervorgerufene Farbreaktion tatsächlich durch aktive Cholesterinoxidase verursacht wird.

Herstellung der Indikatorplatten:

[0026] Für den Plattentest zur Bestimmung von Cholesterinoxidase-Aktivität werden 100 ml 2%ige low-melting-point-Agarose (Sea Plaque BIOzym 50113) aufgeschmolzen und bei einer Temperatur von 42°C eine vorgewärmte Lösung von:

- 48 mg 4-Aminoantipyrin (Boehringer Mannheim GmbH, Katalog-Nr. 073474)
- 306 mg EST (N-Ethyl-N-sulfoethyl-3-methylanilinkaliumsalz (Boehringer Mannheim GmbH, Katalog-Nr. 586854))
- 2,5 mg Meerrettichperoxidase Reinheitsgrad II (ca. 260 U/mg (Boehringer Mannheim GmbH, Katalog-Nr. 005096))
- 60 µl Natriumazidlösung (20%ig)
- 10 ml 1 mol/l Kaliumphosphat pH 7,2
- 150 mg Cholsäurenatriumsalz (Merck, Katalog-Nr. 12448)
- 10 ml Cholesterinsubstratlösung (s. u.)
- H₂O ad 100 ml

zu der aufgeschmolzenen Agarose gegeben, vorsichtig gemischt, jeweils 10 ml in Petrischalen gegossen und zur Aufbewahrung dunkel gehalten.

Cholesterinsubstratlösung:

[0027] 500 mg Cholesterin (Boehringer Mannheim GmbH, Katalog-Nr. 121312) werden in 12,5 ml 1-Propanol (Merck, Katalog-Nr. 997) gelöst, nach Zugabe von 10 g Thesit (Boehringer Mannheim GmbH, Katalog-Nr. 006190) gut gemischt und H₂O ad 100 ml zugegeben. Die Substratlösung kann bei Raumtemperatur mehrere Monate aufbewahrt werden.

Beispiel 2**Charakterisierung des Cholesterinoxidase-Gens**

[0028] Das Plasmid eines gemäß Beispiel 1 erhaltenen Klon (pUC-Chol-B2) wird nach Standardmethoden isoliert und einer Restriktionskartierung mit den Restriktionsendonukleasen BamHI, EcoRI, KpnI, XhoI, PstI unterzogen. Es zeigt sich, daß ein DNA-Fragment aus dem Genom von Brevibacterium in der Größe von ca. 5,5 kb in dem Plasmid pUC-Chol-B2 inseriert ist. Durch Subklonierung verschiedener Teilfragmente dieses 5,5 kb-Stückes und anschließender Bestimmung der Cholesterin-oxidase-Aktivität der erhaltenen E. coli-Klone kann das Cholesterinoxidase-Gen auf ein BamHI-Fragment von 2,3 kb-Größe eingeeengt werden. Das Plasmid mit diesem Fragment wird pUC-Chol-B2-BB genannt (Fig. 1). Die DNA-Sequenz dieses Fragmentes wird bestimmt und auf einem Leseraster, das für Cholesterinoxidase kodiert, hin untersucht. Die Sequenz dieses Leserahmens für die reife Cholesterinoxidase ist in SEQ ID NO 1 wiedergegeben.

Beispiel 3**Konstruktion eines Plasmids zur Expression des Cholesterinoxidase-Gens mit heterologer Signalsequenz**

[0029] Ein Vergleich der N-terminalen Aminosäuresequenz von Cholesterinoxidase, die aus Brevibacterium isoliert wurde, mit dem gesamten für Cholesterinoxidase kodierenden Leseraster von pUC-Chol-B2-BB zeigt, daß im reifen Protein die ersten 52 kodierten Aminosäuren der Gensequenz fehlen. Diese 52 Aminosäuren zeigen die Struktur einer typischen Exportsignalsequenz gram-positiver Prokaryonten (von Heijne, Biochim. Biophys. Acta 947 (1988), 307 - 333). Für die Konstruktion von rekombinanten Cholesterinoxidase-Genen, bei denen diese Signalsequenz gegen andere Sequenzen ersetzt ist, wird zunächst ein 387 bp großes DNA-Fragment aus dem Plasmid pUC-Chol-B2-BB unter Verwendung der in SEQ ID NO 30 und 31 gezeigten Oligonukleotide mittels PCR amplifiziert. Dieses Fragment enthält den für den N-terminalen Teil der reifen Oxidase kodierenden Bereich mit einer neuen SphI-Schnittstelle direkt vor dem N-Terminus der Aminosäuresequenz des reifen Enzyms. Dieses PCR-Fragment wird mit SphI und PstI gespalten und zusammen mit einem PstI EcoRI-Fragment aus pUC-Chol-B2-BB, das den restlichen Anteil des Cholesterinoxidase-Gens enthält, in den mit SphI und EcoRI gespaltenen Expressionsvektor pmgISphI ligiert und so der Vektor pmgI-Chol-SB erhalten. In diesem Vektor enthält das Cholesterinoxidase-Gen eine in E. coli funktionelle Signalsequenz aus Salmonella typhimurium (beschrieben in WO 88/093773).

Beispiel 4

Konstruktion eines Plasmids zur Expression des Cholesterin-oxidase-Gens ohne Signalpeptid-kodierende Sequenz unter Kontrolle des lacUV5-Promotors

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[0030] Aus dem Plasmid pmgl-Chol-SB wird durch Behandlung mit den Restriktionsendonukleasen SphI und BamBI ein DNA-Fragment von ca. 1,85 kb Größe herausgeschnitten, das den gesamten Anteil der kodierenden Sequenz der reifen Cholesterin-oxidase, aber nicht die für das Signal-Peptid kodierende Sequenz enthält. Dieses Fragment wird in den vorher mit SphI und BamBI geschnittenen Plasmidvektor pUC19 eingesetzt. In dem so erhaltenen Plasmid plac-Chol-cyt liegt das Cholesterin-oxidase-Gen im korrekten Leseraster an die ersten zehn Codons des lacZ'-Gens aus pUC19 anfusioniert vor und liegt unter der Kontrolle des lacUV5-Promotors (Fig. 2).

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Beispiel 5

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Konstruktion eines Plasmids zur Expression des Cholesterin-oxidase-Gens ohne Signalpeptid-kodierende Sequenz unter Kontrolle des sauerstoffregulierten pfl-Promotors

[0031] Durch PCR-Technik wird aus dem Plasmid plac_Chol_cyt unter Verwendung der in SEQ ID NO 32 und 33 dargestellten Oligonukleotide ein DNA-Fragment von 432 bp Größe erzeugt, das vor dem ATG-Startcodon eine ClaI-Schnittstelle enthält. Dieses PCR-Fragment wird mit ClaI und PstI geschnitten. Durch Behandlung mit den Restriktionsendonukleasen PstI und BamHI wird aus dem Plasmid plac-Chol-cyt weiterhin ein Fragment mit dem restlichen C-terminalen Anteil des Cholesterin-oxidase-Gens herausgeschnitten. Beide Fragmente werden simultan in den mit BamHI und ClaI gespaltenen Expressionsvektor pfl 20AT1-SD einligiert. Das korrekte Ligationsprodukt enthält nun den Leseraster der reifen Cholesterin-oxidase anfusioniert an die ersten zehn Codons des lacZ'-Gens aus pUC19 unter der Kontrolle des sauerstoffregulierten pfl-Promotors (Fig. 3). Dieses Plasmid trägt die Bezeichnung ppfl-Chol-cyt.

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Beispiel 6

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Konstruktion eines Plasmids zur Expression des Cholesterin-oxidase-Gens mit alternativer N-terminaler Fusionssequenz

[0032] Zur Entfernung der im 3' untranslatierten Bereich des Cholesterin-oxidase-Gens gelegenen SphI-Schnittstelle des Plasmids ppfl-Chol-cyt wird die Plasmid-DNA mit SmaI und EcoRV geschnitten und wieder religiert. 100 ng des so entstandenen Plasmids ppfl-Chol-cyt-Aterm werden dann mit den Restriktionsenzymen ClaI und SphI gespalten. Das entstandene 4,76 kb große DNA-Fragment wird in low-melting-point Agarose elektrophoretisch aufgetrennt, ausgeschnitten und eluiert (Glassmilk®-Kit, Bio 101). 100ng des so gereinigten DNA-Fragments werden mit 50 pmol eines Adapter-Oligonukleotids mit der in SEQ ID NO 34 dargestellten Sequenz (wobei "N" eine äquimolare Mischung aller 4 Basen bedeutet) versetzt und 2 Stunden bei 37°C mit T4-DNA-Ligase behandelt. Anschließend wird der Ansatz mit einer Mischung aus 4 dNTP's (Endkonz. 0,125 mmol/l) versetzt und 40 Minuten bei 37°C mit Klenow-DNA-Polymerase behandelt. Die so erhaltene Plasmid-DNA wird in E. coli XL1-blue (Stratagene) transformiert. Mit Hilfe des in Beispiel 1 beschriebenen Kolonie-Aktivitätstest werden einzelne Kolonien von erhaltenen Klonen bezüglich ihrer Cholesterin-oxidase-Aktivität verglichen. Klone mit hoher Cholesterin-oxidase-Aktivität werden isoliert und die Plasmid-DNA durch Restriktionsanalyse und DNA-Sequenzierung charakterisiert. Für das Plasmid eines Klons mit besonders hoher Cholesterin-oxidase-Aktivität wird die Sequenz SEQ ID NO 23 ermittelt. Das betreffende Plasmid wird ppfl-MSN3H-Chol-cyt-Aterm genannt. Es ist zu erwarten, daß in der dargestellten Art und Weise nach Isolierung und Charakterisierung genügend vieler verschiedener Klone auch noch weitere für eine besonders hohe Expression geeignete Klone gefunden werden können. Zur Wiedervervollständigung des 3'-untranslatierten Anteils wird das Plasmid ppfl-MSN3H-Chol-cyt-Aterm mit ClaI und XhoI geschnitten. Ein DNA-Fragment von ca. 1,1kb mit der Translationsinitiationsregion und dem N-terminalen Anteil des Cholesterin-oxidase-Gens wird isoliert und in das ebenfalls mit ClaI und XhoI geschnittene Plasmid ppfl-Chol-cyt einligiert (Fig. 4). Das erhaltene Plasmid trägt die Bezeichnung ppfl-MSN3H-Chol-cyt.

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Beispiel 7

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Vergleich der Bildung von Cholesterin-oxidase durch die verschiedenen Expressionsplasmide in E. coli

[0033] Die Plasmide pUC-Chol-B2, pUC-Chol-B2-BB, pmgl-Chol-SB, plac-Chol-cyt, ppfl-Chol-cyt, ppfl-MSN3H-Chol-cyt werden jeweils in E. coli K12 XL1-blue transformiert. Zum Vergleich der gebildeten Enzymmenge werden die Klone jeweils 15 Stunden bei 30°C in LB-Medium, das 200 µg/ml Ampicillin und folgende weiteren Zusätze

enthält, angezogen:

Klone mit den Plasmiden pUC-Chol-B2, pUC-Chol-B2-BB, plac-Chol-cyt, bei denen das Cholesterinoxidase-Gen jeweils unter der Kontrolle des lacUV5-Promotors steht, bekommen zusätzlich 1 mmol/l IPTG, der Klon mit dem Plasmid pmgl-Chol-SB mit dem Glucose-reprimierten mgl-Promotor erhält keinen weiteren Zusatz, Klone mit den Plasmiden ppfl-Chol-cyt, ppfl-MSN3H-Chol-cyt mit dem sauerstoffregulierten pfl-Promotor erhalten 0,4% Glucose und werden in Stickstoff begasten verschlossenen Serumflaschen angezogen, wobei das Medium mit KOH auf pH 7,0 eingestellt wurde. Nach erfolgter Anzucht wird die erreichte Zelldichte durch photometrische Messung der Trübung bei 420 nm bestimmt. Die Zellen von 1 ml Kulturbrühe werden dann durch Zentrifugation in einer Mikrozentrifuge bei 10.000 g sedimentiert und wieder in 0,5 ml H₂O bidest resuspendiert. Der Zellaufschluß erfolgt durch 2 x 30 Sekunden Ultraschallbehandlung (Branson Sonifier, Modell 450, Standard-Microtip, Konisch). Die so erhaltenen Zellextrakte werden nach entsprechender Verdünnung in den folgenden Enzymtest eingesetzt: Hierzu werden in Quartz-Küvetten pipettiert: 3 ml Kaliumphosphatpuffer (0,5 mol/l, pH 7,5), der 0,4 % Thesit® (Boehringer Mannheim GmbH, Katalog-Nr. 006190) enthält, 0,1 ml Cholesterinlösung (0,4 % Cholesterin, 10 % 1-Propanol, 10 % Thesit®), 0,02 ml H₂O₂ (0,49 mol/l in bidest. Wasser), es wird gemischt, nach Zugabe von 0,02 ml Katalase (aus Rinderleber, 20 mg Protein/ml, spezifische Aktivität ca. 65.000 U/mg, Boehringer Mannheim GmbH, Katalog-Nr. 0156744 unmittelbar vor Messung mit eiskaltem Kaliumphosphatpuffer, der 0,4 % Thesit enthält, auf 0,075 - 0,15 U/ml verdünnt) erneut gemischt, die Lösung auf eine Temperatur von 25°C gebracht und anschließend die Reaktion durch Zugabe von 0,05 ml Probelösung gestartet. Nach vorsichtigem Mischen wird die Absorptionsänderung bei 240 nm verfolgt und die Aktivität der Cholesterinoxidase aus dem linearen Bereich der Absorptionskurve ermittelt:

$$\text{Aktivität} = \frac{3,19}{\epsilon \cdot 240 \times 0,05 \times 1} \Delta A \text{ min. (U/ml Probelösung)}$$

wobei $\epsilon \cdot 240 = 15,5 \text{ mmol}^{-1} \times 1 \times \text{cm}^{-1}$ ist.

[0034] Die erhaltenen Werte für Zelldichte und Enzymaktivität sind in Tabelle 1 dargestellt.

Tabelle 1

Klon/Plasmid	Zelldichte (E 420)	Units je Zelldichte	Units pro ml
pUC-Chol-B2	7,0	0,007	0,049
pUC-Chol-B2-BB	8,4	0,068	0,571
pmgl-Chol-SB	1,3	0,014	0,018
plac-Chol-cyt	8,6	0,725	6,235
ppfl-Chol-cyt	1,25	1,675	2,094
ppfl-MSN3H-Chol-cyt	3,7	1,463	5,413

[0035] Die erhaltenen Ergebnisse zeigen, daß mit solchen Konstrukten, die eine zytoplasmatische Expression der Cholesterinoxidase bewirken, eine deutlich höhere Aktivität der rekombinant hergestellten Cholesterinoxidase erhalten werden kann als mit solchen Konstrukten, die zu einer Sekretion der rekombinant hergestellten Cholesterinoxidase führen.

SEQUENZPROTOKOLL

[0036]

(1) ALGEMEINE INFORMATION:

(i) ANMELDER:

(A) NAME: Boehringer Mannheim GmbH

(B) STRASSE: Sandhofer Str. 116
(C) ORT: Mannheim
(E) LAND: Deutschland
(F) POSTLEITZAHL: D - 6800

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(ii) ANMELDETITEL: Cholesterinoxidase aus *Brevibacterium sterolicum*

(iii) ANZAHL DER SEQUENZEN: 34

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(iv) COMPUTER-LESBARE FORM:

(A) DATENTRÄGER: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) BETRIEBSSYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPA)

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(2) INFORMATION ZU SEQ ID NO: 1:

(i) SEQUENZ CHARAKTERISTIKA:

20

(A) LANGE: 1683 Basenpaare
(B) ART: Nukleinsäure
(C) STRANGFORM: Einzel
(D) TOPOLOGIE: linear

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(ii) ART DES MOLEKÜLS: DNS (genomisch)

(ix) MERKMALE:

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(A) NAME/SCHLÜSSEL: CDS
(B) LAGE: 1..1683

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 1:

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5	TCG ACC GGG CCG GTC GCG CCG CTT CCG ACG CCG CCG AAC TTC CCG AAC Ser Thr Gly Pro Val Ala Pro Leu Pro Thr Pro Pro Asn Phe Pro Asn 1 5 10 15	48
10	GAC ATC GCG CTG TTC CAG CAG GCG TAC CAG AAC TGG TCC AAG GAG ATC Asp Ile Ala Leu Phe Gln Gln Ala Tyr Gln Asn Trp Ser Lys Glu Ile 20 25 30	96
15	ATG CTG GAC GCC ACT TGG GTC TGC TCG CCC AAG ACG CCG CAG GAT GTC Met Leu Asp Ala Thr Trp Val Cys Ser Pro Lys Thr Pro Gln Asp Val 35 40 45	144
20	GTT CGC CTT GCC AAC TGG GCG CAC GAG CAC GAC TAC AAG ATC CGC CCG Val Arg Leu Ala Asn Trp Ala His Glu His Asp Tyr Lys Ile Arg Pro 50 55 60	192
25	CGC GGC GCG ATG CAC GGC TGG ACC CCG CTC ACC GTG GAG AAG GGG GCC Arg Gly Ala Met His Gly Trp Thr Pro Leu Thr Val Glu Lys Gly Ala 65 70 75 80	240
30	AAC GTC GAG AAG GTG ATC CTC GCC GAC ACG ATG ACG CAT CTG AAC GGC Asn Val Glu Lys Val Ile Leu Ala Asp Thr Met Thr His Leu Asn Gly 85 90 95	288
35	ATC ACG GTG AAC ACG GGC GGC CCC GTG GCT ACC GTC ACC GCC GGT GCC Ile Thr Val Asn Thr Gly Gly Pro Val Ala Thr Val Thr Ala Gly Ala 100 105 110	336
40	GGC GCC AGC ATC GAG GCG ATC GTC ACC GAA CTG CAG AAG CAC GAC CTC Gly Ala Ser Ile Glu Ala Ile Val Thr Glu Leu Gln Lys His Asp Leu 115 120 125	384
45	GGC TGG GCC AAC CTG CCC GCT CCG GGT GTG CTG TCG ATC GGT GGC GCC Gly Trp Ala Asn Leu Pro Ala Pro Gly Val Leu Ser Ile Gly Gly Ala 130 135 140	432
50	CTT GCG GTC AAC GCG CAC GGT GCG GCG CTG CCG GCC GTC GGC CAG ACC Leu Ala Val Asn Ala His Gly Ala Ala Leu Pro Ala Val Gly Gln Thr 145 150 155 160	480
55	ACG CTG CCC GGT CAC ACC TAC GGT TCG CTG AGC AAC CTG GTC ACC GAG Thr Leu Pro Gly His Thr Tyr Gly Ser Leu Ser Asn Leu Val Thr Glu 165 170 175	528
60	CTG ACC GCG GTC GTC TGG AAC GGC ACC ACC TAC GCA CTC GAG ACG TAC Leu Thr Ala Val Val Trp Asn Gly Thr Thr Tyr Ala Leu Glu Thr Tyr 180 185 190	576
65	CAG CGC AAC GAT CCT CGG ATC ACC CCA CTG CTC ACC AAC CTC GGG CGC Gln Arg Asn Asp Pro Arg Ile Thr Pro Leu Leu Thr Asn Leu Gly Arg 195 200 205	624
70	TGC TTC CTG ACC TCG GTG ACG ATG CAG GCC GGC CCC AAC TTC CGT CAG Cys Phe Leu Thr Ser Val Thr Met Gln Ala Gly Pro Asn Phe Arg Gln 210 215 220	672
75	CGG TGC CAG AGC TAC ACC GAC ATC CCG TGG CGG GAA CTG TTC GCG CCG Arg Cys Gln Ser Tyr Thr Asp Ile Pro Trp Arg Glu Leu Phe Ala Pro 225 230 235 240	720

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	AAG GGC GCC GAC GGC CGC ACG TTC GAG AAG TTC GTC GCG GAA TCG GGC	768
	Lys Gly Ala Asp Gly Arg Thr Phe Glu Lys Phe Val Ala Glu Ser Gly	
	245 250 255	
5	GGC GCC GAG GCG ATC TGG TAC CCG TTC ACC GAG AAG CCG TGG ATG AAG	816
	Gly Ala Glu Ala Ile Trp Tyr Pro Phe Thr Glu Lys Pro Trp Met Lys	
	--260 265 270	
10	GTG TGG ACG GTC TCG CCG ACC AAG CCG GAC TCG TCG AAC GAG GTC GGA	864
	Val Trp Thr Val Ser Pro Thr Lys Pro Asp Ser Ser Asn Glu Val Gly	
	275 280 285	
15	AGC CTC GGC TCG GCG GGC TCC CTC GTC GGC AAG CCT CCG CAG GCG CGT	912
	Ser Leu Gly Ser Ala Gly Ser Leu Val Gly Lys Pro Pro Gln Ala Arg	
	290 295 300	
20	GAG GTC TCC GGC CCG TAC AAC TAC ATC TTC TCC GAC AAC CTG CCG GAG	960
	Glu Val Ser Gly Pro Tyr Asn Tyr Ile Phe Ser Asp Asn Leu Pro Glu	
	305 310 315 320	
25	CCC ATC ACC GAC ATG ATC GGC GCC ATC AAC GCC GGA AAC CCC GGA ATC	1008
	Pro Ile Thr Asp Met Ile Gly Ala Ile Asn Ala Gly Asn Pro Gly Ile	
	325 330 335	
30	GCA CCG CTG TTC GGC CCG GCG ATG TAC GAG ATC ACC AAG CTC GGG CTG	1056
	Ala Pro Leu Phe Gly Pro Ala Met Tyr Glu Ile Thr Lys Leu Gly Leu	
	340 345 350	
35	GCC GCG ACG AAT GCC AAC GAC ATC TGG GGC TGG TCG AAG GAC GTC CAG	1104
	Ala Ala Thr Asn Ala Asn Asp Ile Trp Gly Trp Ser Lys Asp Val Gln	
	355 360 365	
40	TTC TAC ATC AAG GCC ACG ACG TTG CGA CTC ACC GAG GGC GGC GGC GCC	1152
	Phe Tyr Ile Lys Ala Thr Thr Leu Arg Leu Thr Glu Gly Gly Gly Ala	
	370 375 380	
45	GTC GTC ACG AGC CGC GCC AAC ATC GCG ACC GTG ATC AAC GAC TTC ACC	1200
	Val Val Thr Ser Arg Ala Asn Ile Ala Thr Val Ile Asn Asp Phe Thr	
	385 390 395 400	
50	GAG TGG TTC CAC GAG CGC ATC GAG TTC TAC CGC GCG AAG GGC GAG TTC	1248
	Glu Trp Phe His Glu Arg Ile Glu Phe Tyr Arg Ala Lys Gly Glu Phe	
	405 410 415	
55	CCG CTC AAC GGT CCG GTC GAG ATC CGC TGC TGC GGG CTC GAT CAG GCA	1296
	Pro Leu Asn Gly Pro Val Glu Ile Arg Cys Cys Gly Leu Asp Gln Ala	
	420 425 430	
60	GCC GAC GTC AAG GTG CCG TCG GTG GGC CCG CCG ACC ATC TCG GCG ACC	1344
	Ala Asp Val Lys Val Pro Ser Val Gly Pro Pro Thr Ile Ser Ala Thr	
	435 440 445	
65	CGT CCG CGT CCG GAT CAT CCG GAC TGG GAC GTC GCG ATC TGG CTG AAC	1392
	Arg Pro Arg Pro Asp His Pro Asp Trp Asp Val Ala Ile Trp Leu Asn	
	450 455 460	
70	GTT CTC GGT GTT CCG GGC ACC CCC GGC ATG TTC GAG TTC TAC CGC GAG	1440
	Val Leu Gly Val Pro Gly Thr Pro Gly Met Phe Glu Phe Tyr Arg Glu	
	465 470 475 480	
75	ATG GAG CAG TGG ATG CGG AGC CAC TAC AAC AAC GAC GAC GCC ACC TTC	1488
	Met Glu Gln Trp Met Arg Ser His Tyr Asn Asn Asp Asp Ala Thr Phe	
	485 490 495	

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	CGG CCC GAG TGG TCG AAG GGG TGG GCG TTC GGT CCC GAC CCG TAC ACC	1536
	Arg Pro Glu Trp Ser Lys Gly Trp Ala Phe Gly Pro Asp Pro Tyr Thr	
	500 505 510	
5	GAC AAC GAC ATC GTC ACG AAC AAG ATG CGC GCC ACC TAC ATC GAA GGT	1584
	Asp Asn Asp Ile Val Thr Asn Lys Met Arg Ala Thr Tyr Ile Glu Gly	
	515 520 525	
10	GTC CCG ACG ACC GAG AAC TGG GAC ACC GCG CGC GCT CCG TAC AAC CAG	1632
	Val Pro Thr Thr Glu Asn Trp Asp Thr Ala Arg Ala Arg Tyr Asn Gln	
	530 535 540	
15	ATC GAC CCG CAT CGC GTG TTC ACC AAC GGA TTC ATG GAC AAG CTG CTT	1680
	Ile Asp Pro His Arg Val Phe Thr Asn Gly Phe Met Asp Lys Leu Leu	
	545 550 555 560	
	CCG	1683
	Pro	

20 (2) INFORMATION ZU SEQ ID NO: 2:

(i) SEQUENZ CHARAKTERISTIKA:

25 (A) LANGE: 561 Aminosäuren
(B) ART: Aminosäure
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Protein

30 (xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 2:

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Ser Thr Gly Pro Val Ala Pro Leu Pro Thr Pro Pro Asn Phe Pro Asn
 1 5 10 15
 5 Asp Ile Ala Leu Phe Gln Gln Ala Tyr Gln Asn Trp Ser Lys Glu Ile
 20 25 30
 Met Leu Asp Ala Thr Trp Val Cys Ser Pro Lys Thr Pro Gln Asp Val
 35 40 45
 10 Val Arg Leu Ala Asn Trp Ala His Glu His Asp Tyr Lys Ile Arg Pro
 50 55 60
 Arg Gly Ala Met His Gly Trp Thr Pro Leu Thr Val Glu Lys Gly Ala
 65 70 75 80
 15 Asn Val Glu Lys Val Ile Leu Ala Asp Thr Met Thr His Leu Asn Gly
 85 90 95
 Ile Thr Val Asn Thr Gly Gly Pro Val Ala Thr Val Thr Ala Gly Ala
 100 105 110
 20 Gly Ala Ser Ile Glu Ala Ile Val Thr Glu Leu Gln Lys His Asp Leu
 115 120 125
 Gly Trp Ala Asn Leu Pro Ala Pro Gly Val Leu Ser Ile Gly Gly Ala
 130 135 140
 25 Leu Ala Val Asn Ala His Gly Ala Ala Leu Pro Ala Val Gly Gln Thr
 145 150 155 160
 Thr Leu Pro Gly His Thr Tyr Gly Ser Leu Ser Asn Leu Val Thr Glu
 165 170 175
 30 Leu Thr Ala Val Val Trp Asn Gly Thr Thr Tyr Ala Leu Glu Thr Tyr
 180 185 190
 Gln Arg Asn Asp Pro Arg Ile Thr Pro Leu Leu Thr Asn Leu Gly Arg
 195 200 205
 35 Cys Phe Leu Thr Ser Val Thr Met Gln Ala Gly Pro Asn Phe Arg Gln
 210 215 220
 Arg Cys Gln Ser Tyr Thr Asp Ile Pro Trp Arg Glu Leu Phe Ala Pro
 225 230 235 240
 Lys Gly Ala Asp Gly Arg Thr Phe Glu Lys Phe Val Ala Glu Ser Gly
 245 250 255
 45 Gly Ala Glu Ala Ile Trp Tyr Pro Phe Thr Glu Lys Pro Trp Met Lys
 260 265 270

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Val Trp Thr Val Ser Pro Thr Lys Pro Asp Ser Ser Asn Glu Val Gly
 275 280 285
 5 Ser Leu Gly Ser Ala Gly Ser Leu Val Gly Lys Pro Pro Gln Ala Arg
 290 295 300
 Glu Val Ser Gly Pro Tyr Asn Tyr Ile Phe Ser Asp Asn Leu Pro Glu
 305 310 315 320
 10 Pro Ile Thr Asp Met Ile Gly Ala Ile Asn Ala Gly Asn Pro Gly Ile
 325 330 335
 Ala Pro Leu Phe Gly Pro Ala Met Tyr Glu Ile Thr Lys Leu Gly Leu
 340 345 350
 15 Ala Ala Thr Asn Ala Asn Asp Ile Trp Gly Trp Ser Lys Asp Val Gln
 355 360 365
 Phe Tyr Ile Lys Ala Thr Thr Leu Arg Leu Thr Glu Gly Gly Gly Ala
 370 375 380
 20 Val Val Thr Ser Arg Ala Asn Ile Ala Thr Val Ile Asn Asp Phe Thr
 385 390 395 400
 Glu Trp Phe His Glu Arg Ile Glu Phe Tyr Arg Ala Lys Gly Glu Phe
 405 410 415
 25 Pro Leu Asn Gly Pro Val Glu Ile Arg Cys Cys Gly Leu Asp Gln Ala
 420 425 430
 Ala Asp Val Lys Val Pro Ser Val Gly Pro Pro Thr Ile Ser Ala Thr
 435 440 445
 30 Arg Pro Arg Pro Asp His Pro Asp Trp Asp Val Ala Ile Trp Leu Asn
 450 455 460
 Val Leu Gly Val Pro Gly Thr Pro Gly Met Phe Glu Phe Tyr Arg Glu
 465 470 475 480
 35 Met Glu Gln Trp Met Arg Ser His Tyr Asn Asn Asp Asp Ala Thr Phe
 485 490 495
 40 Arg Pro Glu Trp Ser Lys Gly Trp Ala Phe Gly Pro Asp Pro Tyr Thr
 500 505 510
 Asp Asn Asp Ile Val Thr Asn Lys Met Arg Ala Thr Tyr Ile Glu Gly
 515 520 525
 45 Val Pro Thr Thr Glu Asn Trp Asp Thr Ala Arg Ala Arg Tyr Asn Gln
 530 535 540
 Ile Asp Pro His Arg Val Phe Thr Asn Gly Phe Met Asp Lys Leu Leu
 545 550 555 560
 50 Pro

55 (2) INFORMATION ZU SEQ ID NO: 3:

(i) SEQUENZ CHARAKTERISTIKA:

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- (A) LÄNGE: 48 Basenpaare
- (B) ART: Nukleinsäure
- (C) STRANGFORM: Einzel
- (D) TOPOLOGIE: linear

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(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 3:

TTCCCGCTCA ACGGTCCGGT CGAGATCCGC TGCTGCGGGC TCGATCAG

48

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(2) INFORMATION ZU SEQ ID NO: 4:

(i) SEQUENZ CHARAKTERISTIKA:

15

- (A) LÄNGE: 48 Basenpaare
- (B) ART: Nukleinsäure
- (C) STRANGFORM: Einzel
- (D) TOPOLOGIE: linear

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(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 4:

GCGATCTGGC TGAACGTTCT CGGTGTTCCG GGCACCCCCG GCATGTTC

48

25

(2) INFORMATION ZU SEQ ID NO: 5:

(i) SEQUENZ CHARAKTERISTIKA:

30

- (A) LÄNGE: 36 Basenpaare
- (B) ART: Nukleinsäure
- (C) STRANGFORM: Einzel
- (D) TOPOLOGIE: linear

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(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 5:

GACGCCACCT TCCGGCCCCG GTGGTCGAAG GGGTGG

36

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(2) INFORMATION ZU SEQ ID NO: 6:

(i) SEQUENZ CHARAKTERISTIKA:

45

- (A) LANGE: 46 Basenpaare
- (B) ART: Nukleinsäure
- (C) STRANGFORM: Einzel
- (D) TOPOLOGIE: linear

50

(ix) MERKMALE:

- (A) NAME/SCHLÜSSEL: CDS
- (B) LAGE: 17..46

55

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 6:

CACACAGGAA ACAGCT ATG ACC ATG ATT ACG CCA AGC TTG CAT GCC
 Met Thr Met Ile Thr Pro Ser Leu His Ala
 1 5 10

46

5

(2) INFORMATION ZU SEQ ID NO: 7:

(i) SEQUENZ CHARAKTERISTIKA:

10

- (A) LÄNGE: 10 Aminosäuren
- (B) ART: Aminosäure
- (D) TOPOLOGIE: linear

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(ii) ART DES KOLEKULS: Protein

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 7:

Met Thr Met Ile Thr Pro Ser Leu His Ala
 1 5 10

20

(2) INFORMATION ZU SEQ ID NO: 8:

(i) SEQUENZ CHARAKTERISTIKA:

25

- (A) LÄNGE: 49 Basenpaare
- (B) ART: Nukleinsäure
- (C) STRANGFORM: Einzel
- (D) TOPOLOGIE: linear

30

(ix) MERKMALE:

- (A) NAME/SCHLUSSEL: CDS
- (B) LÄNGE: 20..49

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(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 8:

GAATTTAAGG GGAACATCG ATG ACC ATG ATT ACG CCA AGC TTG CAT GCC
 Met Thr Met Ile Thr Pro Ser Leu His Ala
 1 5 10

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49

(2) INFORMATION ZU SEQ ID NO: 9:

(i) SEQUENZ CHARAKTERISTIKA:

45

- (A) LÄNGE: 10 Aminosäuren
- (B) ART: Aminosäure
- (D) TOPOLOGIE: linear

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(ii) ART DES MOLEKÜLS: Protein

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 9:

Met Thr Met Ile Thr Pro Ser Leu His Ala
 1 5 10

55

(2) INFORMATION ZU SEQ ID NO: 10:

(i) SEQUENZ CHARAKTERISTIKA:

- 5 (A) LÄNGE: 43 Basenpaare
(B) ART: Nukleinsäure
(C) STRANGFORM: Einzel
(D) TOPOLOGIE: linear

10 (ix) MERKMALE:

- (A) NAME/SCHLÜSSEL: CDS
(B) LÄGE: 20..43

15 (xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 10:

GAATTTAAGG GGAACATCG ATG AGT AAT CAC CAT GGG CAT GCC 43
Met Ser Asn His His Gly His Ala
1 5

(2) INFORMATION ZU SEQ ID NO: 11:

25 (i) SEQUENZ CHARAKTERISTIKA:

- (A) LÄNGE: 8 Aminosäuren
(B) ART: Aminosäure
(D) TOPOLOGIE: linear

30 (ii) ART DES MOLEKÜLS: Protein
(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 11:

35 Met Ser Asn His His Gly His Ala
1 5

(2) INFORMATION ZU SEQ ID NO: 12:

40 (i) SEQUENZ CHARAKTERISTIKA:

- (A) LÄNGE: 45 Basenpaare
(B) ART: Nukleinsäure
(C) STRANGFORM: Einzel
(D) TOPOLOGIE: linear

(ix) MERKMALE:

- 50 (A) NAME/SCHLÜSSEL: CDS
(B) LÄGE: 19..45

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 12:

55 AATTTGGAGG GGAACATT ATG AGT AAT CAT CAC CAT GGG CAT GCC 45
Met Ser Asn His His His Gly His Ala
1 5

(2) INFORMATION ZU SEQ ID NO: 13:

(i) SEQUENZ CHARAKTERISTIKA:

- 5 (A) LÄNGE: 9 Aminosäuren
(B) ART: Aminosäure
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Protein

10 (xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 13:

Met Ser Asn His His His Gly His Ala
1 5

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(2) INFORMATION ZU SEQ ID NO: 14:

(i) SEQUENZ CHARAKTERISTIKA:

- 20 (A) LANGE: 58 Basenpaare
(B) ART: Nukleinsäure
(C) STRANGFORM: Einzel
(D) TOPOLOGIE: linear

25 (ix) MERKMALE:

- (A) NAME/SCHLÜSSEL: CDS
(B) LAGE: 20..58

30 (xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 14:

GAATTTAAGG GGAACATCG ATG AGT AAT ACG CGT AAA CGC AAG CGC CGT ACG 52
Met Ser Asn Thr Arg Lys Arg Lys Arg Arg Thr
1 5 10

35

CAT GCC 58
His Ala

40 (2) INFORMATION ZU SEQ ID NO: 15:

(i) SEQUENZ CHARAKTERISTIKA:

- 45 (A) LÄNGE: 13 Aminosäuren
(B) ART: Aminosäure
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Protein

50 (xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 15:

Met Ser Asn Thr Arg Lys Arg Lys Arg Arg Thr His Ala
1 5 10

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(2) INFORMATION ZU SEQ ID NO: 16:

(i) SEQUENZ CHARAKTERISTIKA:

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	CACACAGGAA ACAGCT ATG ACC ATG ATT ACG CCA AGC TTG CAT GCC TCG	49
	Met Thr Met Ile Thr Pro Ser Leu His Ala Ser	
	1 5 10	
5	ACC GGG CCG GTC GCG CCG CTT CCG ACG CCG CCG AAC TTC CCG AAC GAC	97
	Thr Gly Pro Val Ala Pro Leu Pro Thr Pro Pro Asn Phe Pro Asn Asp	
	15 20 25	
10	ATC GCG CTG TTC CAG CAG GCG TAC CAG AAC TGG TCC AAG GAG ATC ATG	145
	Ile Ala Leu Phe Gln Gln Ala Tyr Gln Asn Trp Ser Lys Glu Ile Met	
	30 35 40	
15	CTG GAC GCC ACT TGG GTC TGC TCG CCC AAG ACG CCG CAG GAT GTC GTT	193
	Leu Asp Ala Thr Trp Val Cys Ser Pro Lys Thr Pro Gln Asp Val Val	
	45 50 55	
20	CGC CTT GCC AAC TGG GCG CAC GAG CAC GAC TAC AAG ATC CGC CCG CGC	241
	Arg Leu Ala Asn Trp Ala His Glu His Asp Tyr Lys Ile Arg Pro Arg	
	60 65 70 75	
25		
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35		
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55		

	GGC	GCG	ATG	CAC	GGC	TGG	ACC	CCG	CTC	ACC	GTG	GAG	AAG	GGG	GCC	AAC	289
	Gly	Ala	Met	His	Gly	Trp	Thr	Pro	Leu	Thr	Val	Glu	Lys	Gly	Ala	Asn	
					80					85					90		
5	GTC	GAG	AAG	GTG	ATC	CTC	GCC	GAC	ACG	ATG	ACG	CAT	CTG	AAC	GGC	ATC	337
	Val	Glu	Lys	Val	Ile	Leu	Ala	Asp	Thr	Met	Thr	His	Leu	Asn	Gly	Ile	
				95					100					105			
10	ACG	GTG	AAC	ACG	GGC	GCG	CCC	GTG	GCT	ACC	GTG	ACC	GCC	GGT	GCC	GGC	385
	Thr	Val	Asn	Thr	Gly	Gly	Pro	Val	Ala	Thr	Val	Thr	Ala	Gly	Ala	Gly	
			110					115					120				
15	GCC	AGC	ATC	GAG	GCG	ATC	GTG	ACC	GAA	CTG	CAG	AAG	CAC	GAC	CTC	GGC	433
	Ala	Ser	Ile	Glu	Ala	Ile	Val	Thr	Glu	Leu	Gln	Lys	His	Asp	Leu	Gly	
			125				130					135					
20	TGG	GCC	AAC	CTG	CCC	GCT	CCG	GGT	GTG	CTG	TCG	ATC	GGT	GGC	GCC	CTT	481
	Trp	Ala	Asn	Leu	Pro	Ala	Pro	Gly	Val	Leu	Ser	Ile	Gly	Gly	Ala	Leu	
	140					145					150					155	
25	GCG	GTC	AAC	GCG	CAC	GGT	GCG	GCG	CTG	CCG	GCC	GTC	GGC	CAG	ACC	ACG	529
	Ala	Val	Asn	Ala	His	Gly	Ala	Ala	Leu	Pro	Ala	Val	Gly	Gln	Thr	Thr	
				160						165					170		
30	CTG	CCC	GGT	CAC	ACC	TAC	GGT	TCG	CTG	AGC	AAC	CTG	GTC	ACC	GAG	CTG	577
	Leu	Pro	Gly	His	Thr	Tyr	Gly	Ser	Leu	Ser	Asn	Leu	Val	Thr	Glu	Leu	
				175					180					185			
35	ACC	GCG	GTC	GTC	TGG	AAC	GGC	ACC	ACC	TAC	GCA	CTC	GAG	ACG	TAC	CAG	625
	Thr	Ala	Val	Val	Trp	Asn	Gly	Thr	Thr	Tyr	Ala	Leu	Glu	Thr	Tyr	Gln	
			190					195					200				
40	CGC	AAC	GAT	CCT	CGG	ATC	ACC	CCA	CTG	CTC	ACC	AAC	CTC	GGG	CGC	TGC	673
	Arg	Asn	Asp	Pro	Arg	Ile	Thr	Pro	Leu	Leu	Thr	Asn	Leu	Gly	Arg	Cys	
		205					210					215					
45	TTC	CTG	ACC	TCG	GTG	ACG	ATG	CAG	GCC	GGC	CCC	AAC	TTC	CGT	CAG	CGG	721
	Phe	Leu	Thr	Ser	Val	Thr	Met	Gln	Ala	Gly	Pro	Asn	Phe	Arg	Gln	Arg	
	220					225					230					235	
50	TGC	CAG	AGC	TAC	ACC	GAC	ATC	CCG	TGG	CGG	GAA	CTG	TTC	GCG	CCG	AAG	769
	Cys	Gln	Ser	Tyr	Thr	Asp	Ile	Pro	Trp	Arg	Glu	Leu	Phe	Ala	Pro	Lys	
				240					245						250		
55	GGC	GCC	GAC	GGC	GCG	ACG	TTC	GAG	AAG	TTC	GTC	GCG	GAA	TCG	GGC	GGC	817
	Gly	Ala	Asp	Gly	Arg	Thr	Phe	Glu	Lys	Phe	Val	Ala	Glu	Ser	Gly	Gly	
			255					260					265				
60	GCC	GAG	GCG	ATC	TGG	TAC	CCG	TTC	ACC	GAG	AAG	CCG	TGG	ATG	AAG	GTG	865
	Ala	Glu	Ala	Ile	Trp	Tyr	Pro	Phe	Thr	Glu	Lys	Pro	Trp	Met	Lys	Val	
			270				275						280				
65	TGG	ACG	GTC	TCG	CCG	ACC	AAG	CCG	GAC	TCG	TCG	AAC	GAG	GTC	GGA	AGC	913
	Trp	Thr	Val	Ser	Pro	Thr	Lys	Pro	Asp	Ser	Ser	Asn	Glu	Val	Gly	Ser	
		285					290					295					
70	CTC	GCC	TCG	GCG	GGC	TCC	CTC	GTC	GGC	AAG	CCT	CCG	CAG	GCG	CGT	GAG	961
	Leu	Gly	Ser	Ala	Gly	Ser	Leu	Val	Gly	Lys	Pro	Pro	Gln	Ala	Arg	Glu	
	300					305					310					315	
75	GTC	TCC	GGC	CCG	TAC	AAC	TAC	ATC	TTC	TCC	GAC	AAC	CTG	CCG	GAG	CCC	1009
	Val	Ser	Gly	Pro	Tyr	Asn	Tyr	Ile	Phe	Ser	Asp	Asn	Leu	Pro	Glu	Pro	
				320					325						330		

5	ATC ACC GAC ATG ATC GGC GCC ATC AAC GCC GGA AAC CCC GGA ATC GCA Ile Thr Asp Met Ile Gly Ala Ile Asn Ala Gly Asn Pro Gly Ile Ala 335 340 345	1057
10	CCG CTG TTC GGC CCG GCG ATG TAC GAG ATC ACC AAG CTC GGG CTG GCC Pro Leu Phe Gly Pro Ala Met Tyr Glu Ile Thr Lys Leu Gly Leu Ala 350 355 360	1105
15	GCG ACG AAT GCC AAC GAC ATC TGG GGC TGG TCG AAG GAC GTC CAG TTC Ala Thr Asn Ala Asn Asp Ile Trp Gly Trp Ser Lys Asp Val Gln Phe 365 370 375	1153
20	TAC ATC AAG GCC ACG ACG TTG CGA CTC ACC GAG GGC GGC GGC GCC GTC Tyr Ile Lys Ala Thr Thr Leu Arg Leu Thr Glu Gly Gly Ala Val 380 385 390 395	1201
25	GTC ACG AGC CGC GCC AAC ATC GCG ACC GTG ATC AAC GAC TTC ACC GAG Val Thr Ser Arg Ala Asn Ile Ala Thr Val Ile Asn Asp Phe Thr Glu 400 405 410	1249
30	TGG TTC CAC GAG CGC ATC GAG TTC TAC CGC GCG AAG GGC GAG TTC CCG Trp Phe His Glu Arg Ile Glu Phe Tyr Arg Ala Lys Gly Glu Phe Pro 415 420 425	1297
35	CTC AAC GGT CCG GTC GAG ATC CGC TGC TGC GGG CTC GAT CAG GCA GCC Leu Asn Gly Pro Val Glu Ile Arg Cys Cys Gly Leu Asp Gln Ala Ala 430 435 440	1345
40	GAC GTC AAG GTG CCG TCG GTG GGC CCG CCG ACC ATC TCG GCG ACC CGT Asp Val Lys Val Pro Ser Val Gly Pro Pro Thr Ile Ser Ala Thr Arg 445 450 455	1393
45	CCG CGT CCG GAT CAT CCG GAC TGG GAC GTC GCG ATC TGG CTG AAC GTT Pro Arg Pro Asp His Pro Asp Trp Asp Val Ala Ile Trp Leu Asn Val 460 465 470 475	1441
50	CTC GGT GTT CCG GGC ACC CCC GGC ATG TTC GAG TTC TAC CGC GAG ATG Leu Gly Val Pro Gly Thr Pro Gly Met Phe Glu Phe Tyr Arg Glu Met 480 485 490	1489
55	GAG CAG TGG ATG CCG AGC CAC TAC AAC AAC GAC GAC GCC ACC TTC CCG Glu Gln Trp Met Arg Ser His Tyr Asn Asn Asp Asp Ala Thr Phe Arg 495 500 505	1537
60	CCC GAG TGG TCG AAG GGG TGG GCG TTC GGT CCC GAC CCG TAC ACC GAC Pro Glu Trp Ser Lys Gly Trp Ala Phe Gly Pro Asp Pro Tyr Thr Asp 510 515 520	1585
65	AAC GAC ATC GTC ACG AAC AAG ATG CGC GCC ACC TAC ATC GAA GGT GTC Asn Asp Ile Val Thr Asn Lys Met Arg Ala Thr Tyr Ile Glu Gly Val 525 530 535	1633
70	CCG ACG ACC GAG AAC TGG GAC ACC GCG CGC GCT CCG TAC AAC CAG ATC Pro Thr Thr Glu Asn Trp Asp Thr Ala Arg Ala Arg Tyr Asn Gln Ile 540 545 550 555	1681
75	GAC CCG CAT CGC GTG TTC ACC AAC GGA TTC ATG GAC AAG CTG CTT CCG Asp Pro His Arg Val Phe Thr Asn Gly Phe Met Asp Lys Leu Leu Pro 560 565 570	1729

(2) INFORMATION ZU SEQ ID NO: 19:

(i) SEQUENZ CHARAKTERISTIKA:

(A) LÄNGE: 571 Aminosäuren

(B) ART: Aminosäure

(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Protein

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 19:

10

Met Thr Met Ile Thr Pro Ser Leu His Ala Ser Thr Gly Pro Val Ala
 1 5 10 15

15

Pro Leu Pro Thr Pro Pro Asn Phe Pro Asn Asp Ile Ala Leu Phe Gln
 20 25 30

Gln Ala Tyr Gln Asn Trp Ser Lys Glu Ile Met Leu Asp Ala Thr Trp
 35 40 45

20

Val Cys Ser Pro Lys Thr Pro Gln Asp Val Val Arg Leu Ala Asn Trp
 50 55 60

Ala His Glu His Asp Tyr Lys Ile Arg Pro Arg Gly Ala Met His Gly
 65 70 75 80

25

Trp Thr Pro Leu Thr Val Glu Lys Gly Ala Asn Val Glu Lys Val Ile
 85 90 95

Leu Ala Asp Thr Met Thr His Leu Asn Gly Ile Thr Val Asn Thr Gly
 100 105 110

30

Gly Pro Val Ala Thr Val Thr Ala Gly Ala Gly Ala Ser Ile Glu Ala
 115 120 125

Ile Val Thr Glu Leu Gln Lys His Asp Leu Gly Trp Ala Asn Leu Pro
 130 135 140

35

Ala Pro Gly Val Leu Ser Ile Gly Gly Ala Leu Ala Val Asn Ala His
 145 150 155 160

Gly Ala Ala Leu Pro Ala Val Gly Gln Thr Thr Leu Pro Gly His Thr
 165 170 175

40

Tyr Gly Ser Leu Ser Asn Leu Val Thr Glu Leu Thr Ala Val Val Trp
 180 185 190

Asn Gly Thr Thr Tyr Ala Leu Glu Thr Tyr Gln Arg Asn Asp Pro Arg
 195 200 205

45

Ile Thr Pro Leu Leu Thr Asn Leu Gly Arg Cys Phe Leu Thr Ser Val
 210 215 220

Thr Met Gln Ala Gly Pro Asn Phe Arg Gln Arg Cys Gln Ser Tyr Thr
 225 230 235 240

50

Asp Ile Pro Trp Arg Glu Leu Phe Ala Pro Lys Gly Ala Asp Gly Arg
 245 250 255

Thr Phe Glu Lys Phe Val Ala Glu Ser Gly Gly Ala Glu Ala Ile Trp
 260 265 270

55

Tyr Pro Phe Thr Glu Lys Pro Trp Met Lys Val Trp Thr Val Ser Pro
 275 280 285
 5 Thr Lys Pro Asp Ser Ser Asn Glu Val Gly Ser Leu Gly Ser Ala Gly
 290 295 300
 Ser Leu Val Gly Lys Pro Pro Gln Ala Arg Glu Val Ser Gly Pro Tyr
 305 310 315 320
 10 Asn Tyr Ile Phe Ser Asp Asn Leu Pro Glu Pro Ile Thr Asp Met Ile
 325 330 335
 Gly Ala Ile Asn Ala Gly Asn Pro Gly Ile Ala Pro Leu Phe Gly Pro
 340 345 350
 15 Ala Met Tyr Glu Ile Thr Lys Leu Gly Leu Ala Ala Thr Asn Ala Asn
 355 360 365
 Asp Ile Trp Gly Trp Ser Lys Asp Val Gln Phe Tyr Ile Lys Ala Thr
 370 375 380
 20 Thr Leu Arg Leu Thr Glu Gly Gly Gly Ala Val Val Thr Ser Arg Ala
 385 390 395 400
 Asn Ile Ala Thr Val Ile Asn Asp Phe Thr Glu Trp Phe His Glu Arg
 405 410 415
 25 Ile Glu Phe Tyr Arg Ala Lys Gly Glu Phe Pro Leu Asn Gly Pro Val
 420 425 430
 Glu Ile Arg Cys Cys Gly Leu Asp Gln Ala Ala Asp Val Lys Val Pro
 435 440 445
 30 Ser Val Gly Pro Pro Thr Ile Ser Ala Thr Arg Pro Arg Pro Asp His
 450 455 460
 Pro Asp Trp Asp Val Ala Ile Trp Leu Asn Val Leu Gly Val Pro Gly
 465 470 475 480
 35 Thr Pro Gly Met Phe Glu Phe Tyr Arg Glu Met Glu Gln Trp Met Arg
 485 490 495
 Ser His Tyr Asn Asn Asp Asp Ala Thr Phe Arg Pro Glu Trp Ser Lys
 500 505 510
 Gly Trp Ala Phe Gly Pro Asp Pro Tyr Thr Asp Asn Asp Ile Val Thr
 515 520 525
 45 Asn Lys Met Arg Ala Thr Tyr Ile Glu Gly Val Pro Thr Thr Glu Asn
 530 535 540
 Trp Asp Thr Ala Arg Ala Arg Tyr Asn Gln Ile Asp Pro His Arg Val
 545 550 555 560
 50 Phe Thr Asn Gly Phe Met Asp Lys Leu Leu Pro
 565 570

(2) INFORMATION ZU SEQ ID NO: 20:

55

(i) SEQUENZ CHARAKTERISTIKA:

(A) LÄNGE: 1732 Basenpaare

(B) ART: Nukleinsäure
(C) STRANGFORM: Einzel
(D) TOPOLOGIE: linear

5 (ix) MERKMALE:

(A) NAME/SCHLÜSSEL: CDS
(B) LAGE: 20..1732

10 (xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 20:

	GAATTTAAGG GGAACATCG ATG ACC ATG ATT ACG CCA AGC TTG CAT GCC TCG	52
	Met Thr Met Ile Thr Pro Ser Leu His Ala Ser	
15	1 5 10	
	ACC GGG CCG GTC GCG CCG CTT CCG ACG CCG CCG AAC TTC CCG AAC GAC	100
	Thr Gly Pro Val Ala Pro Leu Pro Thr Pro Pro Asn Phe Pro Asn Asp	
	15 20 25	
20	ATC GCG CTG TTC CAG CAG GCG TAC CAG AAC TGG TCC AAG GAG ATC ATG	148
	Ile Ala Leu Phe Gln Gln Ala Tyr Gln Asn Trp Ser Lys Glu Ile Met	
	30 35 40	
	CTG GAC GCC ACT TGG GTC TGC TCG CCC AAG ACG CCG CAG GAT GTC GTT	196
25	Leu Asp Ala Thr Trp Val Cys Ser Pro Lys Thr Pro Gln Asp Val Val	
	45 50 55	
	CGC CTT GCC AAC TGG GCG CAC GAG CAC GAC TAC AAG ATC CGC CCG CGC	244
	Arg Leu Ala Asn Trp Ala His Glu His Asp Tyr Lys Ile Arg Pro Arg	
	60 65 70 75	
30	GGC GCG ATG CAC GGC TGG ACC CCG CTC ACC GTG GAG AAG GGC GCC AAC	292
	Gly Ala Met His Gly Trp Thr Pro Leu Thr Val Glu Lys Gly Ala Asn	
	80 85 90	
	GTC GAG AAG GTG ATC CTC GCC GAC ACG ATG ACG CAT CTG AAC GGC ATC	340
35	Val Glu Lys Val Ile Leu Ala Asp Thr Met Thr His Leu Asn Gly Ile	
	95 100 105	
	ACG GTG AAC ACG GGC GGC CCC GTG GCT ACC GTC ACC GCC GGT GCC GGC	388
	Thr Val Asn Thr Gly Gly Pro Val Ala Thr Val Thr Ala Gly Ala Gly	
	110 115 120	
40	GCC AGC ATC GAG GCG ATC GTC ACC GAA CTG CAG AAG CAC GAC CTC GGC	436
	Ala Ser Ile Glu Ala Ile Val Thr Glu Leu Gln Lys His Asp Leu Gly	
	125 130 135	
45	TGG GCC AAC CTG CCC GCT CCG GGT GTG CTG TCG ATC GGT GGC GCC CTT	484
	Trp Ala Asn Leu Pro Ala Pro Gly Val Leu Ser Ile Gly Gly Ala Leu	
	140 145 150 155	
	GCG GTC AAC GCG CAC GGT GCG GCG CTG CCG GCC GTC GGC CAG ACC ACG	532
	Ala Val Asn Ala His Gly Ala Ala Leu Pro Ala Val Gly Gln Thr Thr	
50	160 165 170	
	CTG CCC GGT CAC ACC TAC GGT TCG CTG AGC AAC CTG GTC ACC GAG CTG	580
	Leu Pro Gly His Thr Tyr Gly Ser Leu Ser Asn Leu Val Thr Glu Leu	
	175 180 185	

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	ACC	GCG	GTC	GTC	TGG	AAC	GGC	ACC	ACC	TAC	GCA	CTC	GAG	ACG	TAC	CAG	628
	Thr	Ala	Val	Val	Trp	Asn	Gly	Thr	Thr	Tyr	Ala	Leu	Glu	Thr	Tyr	Gln	
			190				195					200					
5	CGC	AAC	GAT	CCT	CGG	ATC	ACC	CCA	CTG	CTC	ACC	AAC	CTC	GGG	CGC	TGC	676
	Arg	Asn	Asp	Pro	Arg	Ile	Thr	Pro	Leu	Leu	Thr	Asn	Leu	Gly	Arg	Cys	
		205					210					215					
10	TTC	CTG	ACC	TCG	GTG	ACG	ATG	CAG	GCC	GGC	CCC	AAC	TTC	CGT	CAG	CGG	724
	Phe	Leu	Thr	Ser	Val	Thr	Met	Gln	Ala	Gly	Pro	Asn	Phe	Arg	Gln	Arg	
		220				225					230					235	
	TGC	CAG	AGC	TAC	ACC	GAC	ATC	CCG	TGG	CGG	GAA	CTG	TTC	GCG	CCG	AAG	772
	Cys	Gln	Ser	Tyr	Thr	Asp	Ile	Pro	Trp	Arg	Glu	Leu	Phe	Ala	Pro	Lys	
					240					245					250		
15	GGC	GCC	GAC	GGC	CGC	ACG	TTC	GAG	AAG	TTC	GTC	GCG	GAA	TCG	GGC	GGC	820
	Gly	Ala	Asp	Gly	Arg	Thr	Phe	Glu	Lys	Phe	Val	Ala	Glu	Ser	Gly	Gly	
				255					260					265			
20	GCC	GAG	GCG	ATC	TGG	TAC	CCG	TTC	ACC	GAG	AAG	CCG	TGG	ATG	AAG	GTG	868
	Ala	Glu	Ala	Ile	Trp	Tyr	Pro	Phe	Thr	Glu	Lys	Pro	Trp	Met	Lys	Val	
			270					275					280				
	TGG	ACG	GTC	TCG	CCG	ACC	AAG	CCG	GAC	TCG	TCG	AAC	GAG	GTC	GGA	AGC	916
	Trp	Thr	Val	Ser	Pro	Thr	Lys	Pro	Asp	Ser	Ser	Asn	Glu	Val	Gly	Ser	
		285					290					295					
25	CTC	GGC	TCG	GCG	GGC	TCC	CTC	GTC	GGC	AAG	CCT	CCG	CAG	GCG	CGT	GAG	964
	Leu	Gly	Ser	Ala	Gly	Ser	Leu	Val	Gly	Lys	Pro	Pro	Gln	Ala	Arg	Glu	
		300				305					310					315	
30	GTC	TCC	GGC	CCG	TAC	AAC	TAC	ATC	TTC	TCC	GAC	AAC	CTG	CCG	GAG	CCC	1012
	Val	Ser	Gly	Pro	Tyr	Asn	Tyr	Ile	Phe	Ser	Asp	Asn	Leu	Pro	Glu	Pro	
					320					325					330		
	ATC	ACC	GAC	ATG	ATC	GGC	GCC	ATC	AAC	GCC	GGA	AAC	CCC	GGA	ATC	GCA	1060
	Ile	Thr	Asp	Met	Ile	Gly	Ala	Ile	Asn	Ala	Gly	Asn	Pro	Gly	Ile	Ala	
					335				340					345			
35	CCG	CTG	TTC	GGC	CCG	GCG	ATG	TAC	GAG	ATC	ACC	AAG	CTC	GGG	CTG	GCC	1108
	Pro	Leu	Phe	Gly	Pro	Ala	Met	Tyr	Glu	Ile	Thr	Lys	Leu	Gly	Leu	Ala	
			350					355					360				
40	GCG	ACG	AAT	GCC	AAC	GAC	ATC	TGG	GGC	TGG	TCG	AAG	GAC	GTC	CAG	TTC	1156
	Ala	Thr	Asn	Ala	Asn	Asp	Ile	Trp	Gly	Trp	Ser	Lys	Asp	Val	Gln	Phe	
			365				370					375					
	TAC	ATC	AAG	GCC	ACG	ACG	TTC	CGA	CTC	ACC	GAG	GGC	GGC	GGC	GCC	GTC	1204
	Tyr	Ile	Lys	Ala	Thr	Thr	Leu	Arg	Leu	Thr		Gly	Gly	Gly	Ala	Val	
		380				385					390					395	
	GTC	ACG	AGC	CGC	GCC	AAC	ATC	GCG	ACC	GTG	ATC	AAC	GAC	TTC	ACC	GAG	1252
	Val	Thr	Ser	Arg	Ala	Asn	Ile	Ala	Thr	Val	Ile	Asn	Asp	Phe	Thr	Glu	
					400					405					410		
50	TGG	TTC	CAC	GAG	CGC	ATC	GAG	TTC	TAC	CGC	GCG	AAG	GGC	GAG	TTC	CCG	1300
	Trp	Phe	His	Glu	Arg	Ile	Glu	Phe	Tyr	Arg	Ala	Lys	Gly	Glu	Phe	Pro	
				415					420					425			
55	CTC	AAC	GGT	CCG	GTC	GAG	ATC	CGC	TGC	TGC	GGG	CTC	GAT	CAG	GCA	GCC	1348
	Leu	Asn	Gly	Pro	Val	Glu	Ile	Arg	Cys	Cys	Gly	Leu	Asp	Gln	Ala	Ala	
			430					435					440				

5	GAC GTC AAG GTG CCG TCG GTG GGC CCG CCG ACC ATC TCG GCG ACC CGT Asp Val Lys Val Pro Ser Val Gly Pro Pro Thr Ile Ser Ala Thr Arg 445 450 455	1396
10	CCG CGT CCG GAT CAT CCG GAC TGG GAC GTC GCG ATC TGG CTG AAC GTT Pro Arg Pro Asp His Pro Asp Trp Asp Val Ala Ile Trp Leu Asn Val 460 465 470 475	1444
15	CTC GGT GTT CCG GGC ACC CCC GGC ATG TTC GAG TTC TAC CGC GAG ATG Leu Gly Val Pro Gly Thr Pro Gly Met Phe Glu Phe Tyr Arg Glu Met 480 485 490	1492
20	GAG CAG TGG ATG CCG AGC CAC TAC AAC AAC GAC GAC GCC ACC TTC CGG Glu Gln Trp Met Arg Ser His Tyr Asn Asn Asp Asp Ala Thr Phe Arg 495 500 505	1540
25	CCC GAG TGG TCG AAG GGG TGG GCG TTC GGT CCC GAC CCG TAC ACC GAC Pro Glu Trp Ser Lys Gly Trp Ala Phe Gly Pro Asp Pro Tyr Thr Asp 510 515 520	1588
30	AAC GAC ATC GTC ACG AAC AAG ATG CGC GCC ACC TAC ATC GAA GGT GTC Asn Asp Ile Val Thr Asn Lys Met Arg Ala Thr Tyr Ile Glu Gly Val 525 530 535	1636
35	CCG ACG ACC GAG AAC TGG GAC ACC GCG CGC GCT CGG TAC AAC CAG ATC Pro Thr Thr Glu Asn Trp Asp Thr Ala Arg Ala Arg Tyr Asn Gln Ile 540 545 550 555	1684
40	GAC CCG CAT CGC GTG TTC ACC AAC GGA TTC ATG GAC AAG CTG CTT CCG Asp Pro His Arg Val Phe Thr Asn Gly Phe Met Asp Lys Leu Leu Pro 560 565 570	1732

(2) INFORMATION ZU SEQ ID NO: 21:

(i) SEQUENZ CHARAKTERISTIKA:

- (A) LANGE: 571 Aminosäuren
- (B) ART: Aminosäure
- (D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Protein

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 21:

EP 0 698 102 B1

	Met	Thr	Met	Ile	Thr	Pro	Ser	Leu	His	Ala	Ser	Thr	Gly	Pro	Val	Ala	
	1				5					10					15		
5	Pro	Leu	Pro	Thr	Pro	Pro	Asn	Phe	Pro	Asn	Asp	Ile	Ala	Leu	Phe	Gln	
				20					25					30			
	Gln	Ala	Tyr	Gln	Asn	Trp	Ser	Lys	Glu	Ile	Met	Leu	Asp	Ala	Thr	Trp	
			35					40					45				
10	Val	Cys	Ser	Pro	Lys	Thr	Pro	Gln	Asp	Val	Val	Arg	Leu	Ala	Asn	Trp	
		50					55					60					
	Ala	His	Glu	His	Asp	Tyr	Lys	Ile	Arg	Pro	Arg	Gly	Ala	Met	His	Gly	
	65					70					75					80	
15	Trp	Thr	Pro	Leu	Thr	Val	Glu	Lys	Gly	Ala	Asn	Val	Glu	Lys	Val	Ile	
					85					90					95		

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EP 0 698 102 B1

Leu Ala Asp Thr Met Thr His Leu Asn Gly Ile Thr Val Asn Thr Gly
 100 105 110
 5 Gly Pro Val Ala Thr Val Thr Ala Gly Ala Gly Ala Ser Ile Glu Ala
 115 120 125
 Ile Val Thr Glu Leu Gln Lys His Asp Leu Gly Trp Ala Asn Leu Pro
 130 135 140
 10 Ala Pro Gly Val Leu Ser Ile Gly Gly Ala Leu Ala Val Asn Ala His
 145 150 155 160
 Gly Ala Ala Leu Pro Ala Val Gly Gln Thr Thr Leu Pro Gly His Thr
 165 170 175
 15 Tyr Gly Ser Leu Ser Asn Leu Val Thr Glu Leu Thr Ala Val Val Trp
 180 185 190
 Asn Gly Thr Thr Tyr Ala Leu Glu Thr Tyr Gln Arg Asn Asp Pro Arg
 195 200 205
 20 Ile Thr Pro Leu Leu Thr Asn Leu Gly Arg Cys Phe Leu Thr Ser Val
 210 215 220
 Thr Met Gln Ala Gly Pro Asn Phe Arg Gln Arg Cys Gln Ser Tyr Thr
 225 230 235 240
 25 Asp Ile Pro Trp Arg Glu Leu Phe Ala Pro Lys Gly Ala Asp Gly Arg
 245 250 255
 Thr Phe Glu Lys Phe Val Ala Glu Ser Gly Gly Ala Glu Ala Ile Trp
 260 265 270
 30 Tyr Pro Phe Thr Glu Lys Pro Trp Met Lys Val Trp Thr Val Ser Pro
 275 280 285
 Thr Lys Pro Asp Ser Ser Asn Glu Val Gly Ser Leu Gly Ser Ala Gly
 290 295 300
 35 Ser Leu Val Gly Lys Pro Pro Gln Ala Arg Glu Val Ser Gly Pro Tyr
 305 310 315 320
 Asn Tyr Ile Phe Ser Asp Asn Leu Pro Glu Pro Ile Thr Asp Met Ile
 325 330 335
 40 Gly Ala Ile Asn Ala Gly Asn Pro Gly Ile Ala Pro Leu Phe Gly Pro
 340 345 350
 Ala Met Tyr Glu Ile Thr Lys Leu Gly Leu Ala Ala Thr Asn Ala Asn
 355 360 365
 45 Asp Ile Trp Gly Trp Ser Lys Asp Val Gln Phe Tyr Ile Lys Ala Thr
 370 375 380
 Thr Leu Arg Leu Thr Glu Gly Gly Gly Ala Val Val Thr Ser Arg Ala
 385 390 395 400
 50 Asn Ile Ala Thr Val Ile Asn Asp Phe Thr Glu Trp Phe His Glu Arg
 405 410 415
 55 Ile Glu Phe Tyr Arg Ala Lys Gly Glu Phe Pro Leu Asn Gly Pro Val
 420 425 430

Glu Ile Arg Cys Cys Gly Leu Asp Gln Ala Ala Asp Val Lys Val Pro
 435 440 445
 5 Ser Val Gly Pro Pro Thr Ile Ser Ala Thr Arg Pro Arg Pro Asp His
 450 455 460
 Pro Asp Trp Asp Val Ala Ile Trp Leu Asn Val Leu Gly Val Pro Gly
 465 470 475 480
 10 Thr Pro Gly Met Phe Glu Phe Tyr Arg Glu Met Glu Gln Trp Met Arg
 485 490 495
 Ser His Tyr Asn Asn Asp Asp Ala Thr Phe Arg Pro Glu Trp Ser Lys
 500 505 510
 15 Gly Trp Ala Phe Gly Pro Asp Pro Tyr Thr Asp Asn Asp Ile Val Thr
 515 520 525
 Asn Lys Met Arg Ala Thr Tyr Ile Glu Gly Val Pro Thr Thr Glu Asn
 530 535 540
 20 Trp Asp Thr Ala Arg Ala Arg Tyr Asn Gln Ile Asp Pro His Arg Val
 545 550 555 560
 25 Phe Thr Asn Gly Phe Met Asp Lys Leu Leu Pro
 565 570

(2) INFORMATION ZU SEQ ID NO: 22:

(i) SEQUENZ CHARAKTERISTIKA:

- (A) LÄNGE: 1726 Basenpaare
- (B) ART: Nukleinsäure
- (C) STRANGFORM: Einzel
- (D) TOPOLOGIE: linear

(ix) MERKMALE:

- (A) NAME/SCHLÜSSEL: CDS
- (B) LAGE: 20..1726

(xi) SEQUENZ BESCHREIBUNG: SEQ ID NO: 22:

EP 0 698 102 B1

	GAATTTAAGG GGAACATCG ATG AGT AAT CAC CAT GGG CAT GCC TCG ACC GGG	52
	Met Ser Asn His His Gly His Ala Ser Thr Gly	
	1 5 10	
5	CCG GTC GCG CCG CTT CCG ACG CCG CCG AAC TTC CCG AAC GAC ATC GCG	100
	Pro Val Ala Pro Leu Pro Thr Pro Pro Asn Phe Pro Asn Asp Ile Ala	
	15 20 25	
10	CTG TTC CAG CAG GCG TAC CAG AAC TGG TCC AAG GAG ATC ATG CTG GAC	148
	Leu Phe Gln Gln Ala Tyr Gln Asn Trp Ser Lys Glu Ile Met Leu Asp	
	30 35 40	
	GCC ACT TGG GTC TGC TCG CCC AAG ACG CCG CAG GAT GTC GTT CGC CTT	196
	Ala Thr Trp Val Cys Ser Pro Lys Thr Pro Gln Asp Val Val Arg Leu	
	45 50 55	
15	GCC AAC TGG GCG CAC GAG CAC GAC TAC AAG ATC CGC CCG CGC GGC GCG	244
	Ala Asn Trp Ala His Glu His Asp Tyr Lys Ile Arg Pro Arg Gly Ala	
	60 65 70 75	
20		
25		
30		
35		
40		
45		
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EP 0 698 102 B1

	ATG CAC GGC TGG ACC CCG CTC ACC GTG GAG AAG GGG GCC AAC GTC GAG	292
	Met His Gly Trp Thr Pro Leu Thr Val Glu Lys Gly Ala Asn Val Glu	
	80 85 90	
5	AAG GTG ATC CTC GCC GAC ACG ATG ACG CAT CTG AAC GGC ATC ACG GTG	340
	Lys Val Ile Leu Ala Asp Thr Met Thr His Leu Asn Gly Ile Thr Val	
	95 100 105	
10	AAC ACG GGC GGC CCC GTG GCT ACC GTC ACC GCC GGT GCC GGC GCC AGC	388
	Asn Thr Gly Gly Pro Val Ala Thr Val Thr Ala Gly Ala Gly Ala Ser	
	110 115 120	
15	ATC GAG GCG ATC GTC ACC GAA CTG CAG AAG CAC GAC CTC GGC TGG GCC	436
	Ile Glu Ala Ile Val Thr Glu Leu Gln Lys His Asp Leu Gly Trp Ala	
	125 130 135	
20	AAC CTG CCC GCT CCG GGT GTG CTG TCG ATC GGT GGC GCC CTT GCG GTC	484
	Asn Leu Pro Ala Pro Gly Val Leu Ser Ile Gly Gly Ala Leu Ala Val	
	140 145 150 155	
25	AAC GCG CAC GGT GCG GCG CTG CCG GCC GTC GGC CAG ACC ACG CTG CCC	532
	Asn Ala His Gly Ala Ala Leu Pro Ala Val Gly Gln Thr Thr Leu Pro	
	160 165 170	
30	GGT CAC ACC TAC GGT TCG CTG AGC AAC CTG GTC ACC GAG CTG ACC GCG	580
	Gly His Thr Tyr Gly Ser Leu Ser Asn Leu Val Thr Glu Leu Thr Ala	
	175 180 185	
35	GTC GTC TGG AAC GGC ACC ACC TAC GCA CTC GAG ACG TAC CAG CGC AAC	628
	Val Val Trp Asn Gly Thr Thr Tyr Ala Leu Glu Thr Tyr Gln Arg Asn	
	190 195 200	
40	GAT CCT CGG ATC ACC CCA CTG CTC ACC AAC CTC GGG GGC TGC TTC CTG	676
	Asp Pro Arg Ile Thr Pro Leu Leu Thr Asn Leu Gly Arg Cys Phe Leu	
	205 210 215	
45	ACC TCG GTG ACG ATG CAG GCC GGC CCC AAC TTC CGT CAG CGG TGC CAG	724
	Thr Ser Val Thr Met Gln Ala Gly Pro Asn Phe Arg Gln Arg Cys Gln	
	220 225 230 235	
50	AGC TAC ACC GAC ATC CCG TGG CCG GAA CTG TTC GCG CCG AAG GGC GCC	772
	Ser Tyr Thr Asp Ile Pro Trp Arg Glu Leu Phe Ala Pro Lys Gly Ala	
	240 245 250	
55	GAC GGC CGC ACG TTC GAG AAG TTC GTC GCG GAA TCG GGC GGC GCC GAG	820
	Asp Gly Arg Thr Phe Glu Lys Phe Val Ala Glu Ser Gly Gly Ala Glu	
	255 260 265	
60	GCG ATC TGG TAC CCG TTC ACC GAG AAG CCG TGG ATG AAG GTG TGG ACG	868
	Ala Ile Trp Tyr Pro Phe Thr Glu Lys Pro Trp Met Lys Val Trp Thr	
	270 275 280	
65	GTC TCG CCG ACC AAG CCG GAC TCG TCG AAC GAG GTC GGA AGC CTC GGC	916
	Val Ser Pro Thr Lys Pro Asp Ser Ser Asn Glu Val Gly Ser Leu Gly	
	285 290 295	
70	TCG GCG GGC TCC CTC GTC GGC AAG CCT CCG CAG GCG CGT GAG GTC TCC	964
	Ser Ala Gly Ser Leu Val Gly Lys Pro Pro Gln Ala Arg Glu Val Ser	
	300 305 310 315	
75	GGC CCG TAC AAC TAC ATC TTC TCC GAC AAC CTG CCG GAG CCC ATC ACC	1012
	Gly Pro Tyr Asn Tyr Ile Phe Ser Asp Asn Leu Pro Glu Pro Ile Thr	
	320 325 330	

5	GAC ATG ATC GGC GCC ATC AAC GCC GGA AAC CCC GGA ATC GCA CCG CTG Asp Met Ile Gly Ala Ile Asn Ala Gly Asn Pro Gly Ile Ala Pro Leu 335 340 345	1060
10	TTC GGC CCG GCG ATG TAC GAG ATC ACC AAG CTC GGG CTG GCC GCG ACG Phe Gly Pro Ala Met Tyr Glu Ile Thr Lys Leu Gly Leu Ala Ala Thr 350 355 360	1108
15	AAT GCC AAC GAC ATC TGG GGC TGG TCG AAG GAC GTC CAG TTC TAC ATC Asn Ala Asn Asp Ile Trp Gly Trp Ser Lys Asp Val Gln Phe Tyr Ile 365 370 375	1156
20	AAG GCC ACG ACG TTG CGA CTC ACC GAG GGC GGC GGC GCC GTC GTC ACG Lys Ala Thr Thr Leu Arg Leu Thr Glu Gly Gly Gly Ala Val Val Thr 380 385 390 395	1204
25	AGC CGC GCC AAC ATC GCG ACC GTG ATC AAC GAC TTC ACC GAG TGG TTC Ser Arg Ala Asn Ile Ala Thr Val Ile Asn Asp Phe Thr Glu Trp Phe 400 405 410	1252
30	CAC GAG CGC ATC GAG TTC TAC CGC GCG AAG GGC GAG TTC CCG CTC AAC His Glu Arg Ile Glu Phe Tyr Arg Ala Lys Gly Glu Phe Pro Leu Asn 415 420 425	1300
35	GGT CCG GTC GAG ATC CGC TGC TGC GGG CTC GAT CAG GCA GCC GAC GTC Gly Pro Val Glu Ile Arg Cys Cys Gly Leu Asp Gln Ala Ala Asp Val 430 435 440	1348
40	AAG GTG CCG TCG GTG GGC CCG CCG ACC ATC TCG GCG ACC CGT CCG CGT Lys Val Pro Ser Val Gly Pro Pro Thr Ile Ser Ala Thr Arg Pro Arg 445 450 455	1396
45	CCG GAT CAT CCG GAC TGG GAC GTC GCG ATC TGG CTG AAC GTT CTC GGT Pro Asp His Pro Asp Trp Asp Val Ala Ile Trp Leu Asn Val Leu Gly 460 465 470 475	1444
50	GTT CCG GGC ACC CCC GGC ATG TTC GAG TTC TAC CGC GAG ATG GAG CAG Val Pro Gly Thr Pro Gly Met Phe Glu Phe Tyr Arg Glu Met Glu Gln 480 485 490	1492
55	TGG ATG CGG AGC CAC TAC AAC AAC GAC GAC GCC ACC TTC CGG CCC GAG Trp Met Arg Ser His Tyr Asn Asn Asp Asp Ala Thr Phe Arg Pro Glu 495 500 505	1540
60	TGG TCG AAG GGG TGG GCG TTC GGT CCC GAC CCG TAC ACC GAC AAC GAC Trp Ser Lys Gly Trp Ala Phe Gly Pro Asp Pro Tyr Thr Asp Asn Asp 510 515 520	1588
65	ATC GTC ACG AAC AAG ATG CGC GCC ACC TAC ATC GAA GGT GTC CCG ACG Ile Val Thr Asn Lys Met Arg Ala Thr Tyr Ile Glu Gly Val Pro Thr 525 530 535	1636
70	ACC GAG AAC TGG GAC ACC GCG CGC GCT CGG TAC AAC CAG ATC GAC CCG Thr Glu Asn Trp Asp Thr Ala Arg Ala Arg Tyr Asn Gln Ile Asp Pro 540 545 550 555	1684
75	CAT CGC GTG TTC ACC AAC GGA TTC ATG GAC AAG CTG CTT CCG His Arg Val Phe Thr Asn Gly Phe Met Asp Lys Leu Leu Pro 560 565	1726

(2) INFORMATION ZU SEQ ID NO: 23:

(i) SEQUENZ CHARAKTERISTIKA:

EP 0 698 102 B1

(A) LANGE: 569 Aminosäuren

(B) ART: Aminosäure

(D) TOPOLOGIE: linear

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(ii) ART DES MOLEKÜLS: Protein

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 23:

10 Met Ser Asn His His Gly His Ala Ser Thr Gly Pro Val Ala Pro Leu
 1 5 10 15
 Pro Thr Pro Pro Asn Phe Pro Asn Asp Ile Ala Leu Phe Gln Gln Ala
 20 25 30
 15 Tyr Gln Asn Trp Ser Lys Glu Ile Met Leu Asp Ala Thr Trp Val Cys
 35 40 45
 Ser Pro Lys Thr Pro Gln Asp Val Val Arg Leu Ala Asn Trp Ala His
 50 55 60
 20 Glu His Asp Tyr Lys Ile Arg Pro Arg Gly Ala Met His Gly Trp Thr
 65 70 75 80
 Pro Leu Thr Val Glu Lys Gly Ala Asn Val Glu Lys Val Ile Leu Ala
 85 90 95
 25 Asp Thr Met Thr His Leu Asn Gly Ile Thr Val Asn Thr Gly Gly Pro
 100 105 110
 Val Ala Thr Val Thr Ala Gly Ala Gly Ala Ser Ile Glu Ala Ile Val
 115 120 125
 30 Thr Glu Leu Gln Lys His Asp Leu Gly Trp Ala Asn Leu Pro Ala Pro
 130 135 140
 Gly Val Leu Ser Ile Gly Gly Ala Leu Ala Val Asn Ala His Gly Ala
 145 150 155 160
 35 Ala Leu Pro Ala Val Gly Gln Thr Thr Leu Pro Gly His Thr Tyr Gly
 165 170 175
 Ser Leu Ser Asn Leu Val Thr Glu Leu Thr Ala Val Val Trp Asn Gly
 180 185 190
 40 Thr Thr Tyr Ala Leu Glu Thr Tyr Gln Arg Asn Asp Pro Arg Ile Thr
 195 200 205
 Pro Leu Leu Thr Asn Leu Gly Arg Cys Phe Leu Thr Ser Val Thr Met
 210 215 220
 45 Gln Ala Gly Pro Asn Phe Arg Gln Arg Cys Gln Ser Tyr Thr Asp Ile
 225 230 235 240
 Pro Trp Arg Glu Leu Phe Ala Pro Lys Gly Ala Asp Gly Arg Thr Phe
 245 250 255
 50 Glu Lys Phe Val Ala Glu Ser Gly Gly Ala Glu Ala Ile Trp Tyr Pro
 260 265 270

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Phe Thr Glu Lys Pro Trp Met Lys Val Trp Thr Val Ser Pro Thr Lys
 275 280 285
 5 Pro Asp Ser Ser Asn Glu Val Gly Ser Leu Gly Ser Ala Gly Ser Leu
 290 295 300
 Val Gly Lys Pro Pro Gln Ala Arg Glu Val Ser Gly Pro Tyr Asn Tyr
 305 310 315 320
 10 Ile Phe Ser Asp Asn Leu Pro Glu Pro Ile Thr Asp Met Ile Gly Ala
 325 330 335
 Ile Asn Ala Gly Asn Pro Gly Ile Ala Pro Leu Phe Gly Pro Ala Met
 340 345 350
 15 Tyr Glu Ile Thr Lys Leu Gly Leu Ala Ala Thr Asn Ala Asn Asp Ile
 355 360 365
 Trp Gly Trp Ser Lys Asp Val Gln Phe Tyr Ile Lys Ala Thr Thr Leu
 370 375 380
 20 Arg Leu Thr Glu Gly Gly Ala Val Val Thr Ser Arg Ala Asn Ile
 385 390 395 400
 Ala Thr Val Ile Asn Asp Phe Thr Glu Trp Phe His Glu Arg Ile Glu
 405 410 415
 25 Phe Tyr Arg Ala Lys Gly Glu Phe Pro Leu Asn Gly Pro Val Glu Ile
 420 425 430
 Arg Cys Cys Gly Leu Asp Gln Ala Ala Asp Val Lys Val Pro Ser Val
 435 440 445
 Gly Pro Pro Thr Ile Ser Ala Thr Arg Pro Arg Pro Asp His Pro Asp
 450 455 460
 30 Trp Asp Val Ala Ile Trp Leu Asn Val Leu Gly Val Pro Gly Thr Pro
 465 470 475 480
 Gly Met Phe Glu Phe Tyr Arg Glu Met Glu Gln Trp Met Arg Ser His
 485 490 495
 40 Tyr Asn Asn Asp Asp Ala Thr Phe Arg Pro Glu Trp Ser Lys Gly Trp
 500 505 510
 Ala Phe Gly Pro Asp Pro Tyr Thr Asp Asn Asp Ile Val Thr Asn Lys
 515 520 525
 45 Met Arg Ala Thr Tyr Ile Glu Gly Val Pro Thr Thr Glu Asn Trp Asp
 530 535 540
 Thr Ala Arg Ala Arg Tyr Asn Gln Ile Asp Pro His Arg Val Phe Thr
 545 550 555 560
 50 Asn Gly Phe Met Asp Lys Leu Leu Pro
 565

55 (2) INFORMATION ZU SEQ ID NO: 24:

(i) SEQUENZ CHARAKTERISTIKA:

EP 0 698 102 B1

(A) LÄNGE: 1728 Basenpaare
(B) ART: Nukleinsäure
(C) STRANGFORM: Einzel
(D) TOPOLOGIE: linear

(ix) MERKMALE:

(A) NAME/SCHLÜSSEL: CDS
(B) LAGE: 19..1728

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 24:

15	AATTTGGAGG GGAACATT ATG AGT AAT CAT CAC CAT GGG CAT GCC TCG ACC	51
	Met Ser Asn His His His Gly His Ala Ser Thr	
	1 5 10	
20	GGG CCG GTC GCG CCG CTT CCG ACG CCG CCG AAC TTC CCG AAC GAC ATC	99
	Gly Pro Val Ala Pro Leu Pro Thr Pro Asn Phe Pro Asn Asp Ile	
	15 20 25	
25	GCG CTG TTC CAG CAG GCG TAC CAG AAC TGG TCC AAG GAG ATC ATG CTG	147
	Ala Leu Phe Gln Gln Ala Tyr Gln Asn Trp Ser Lys Glu Ile Met Leu	
	30 35 40	
30	GAC GCC ACT TGG GTC TGC TCG CCC AAG ACG CCG CAG GAT GTC GTT CGC	195
	Asp Ala Thr Trp Val Cys Ser Pro Lys Thr Pro Gln Asp Val Val Arg	
	45 50 55	
35	CTT GCC AAC TGG GCG CAC GAG CAC GAC TAC AAG ATC CGC CCG CGC GGC	243
	Leu Ala Asn Trp Ala His Glu His Asp Tyr Lys Ile Arg Pro Arg Gly	
	60 65 70 75	
40	GCG ATG CAC GGC TGG ACC CCG CTC ACC GTG GAG AAG GGG GCC AAC GTC	291
	Ala Met His Gly Trp Thr Pro Leu Thr Val Glu Lys Gly Ala Asn Val	
	80 85 90	
45	GAG AAG GTG ATC CTC GCC GAC ACG ATG ACG CAT CTG AAC GGC ATC ACG	339
	Glu Lys Val Ile Leu Ala Asp Thr Met Thr His Leu Asn Gly Ile Thr	
	95 100 105	
50	GTG AAC ACG GGC GGC CCC GTG GCT ACC GTC ACC GCC GGT GCC GGC GCC	387
	Val Asn Thr Gly Gly Pro Val Ala Thr Val Thr Ala Gly Ala Gly Ala	
	110 115 120	
55	AGC ATC GAG GCG ATC GTC ACC GAA CTG CAG AAG CAC GAC CTC GGC TGG	435
	Ser Ile Glu Ala Ile Val Thr Glu Leu Gln Lys His Asp Leu Gly Trp	
	125 130 135	
60	GCC AAC CTG CCC GCT CCG GGT GTG CTG TCG ATC GGT GGC GCC CTT GCG	483
	Ala Asn Leu Pro Ala Pro Gly Val Leu Ser Ile Gly Gly Ala Leu Ala	
	140 145 150 155	
65	GTC AAC GCG CAC GGT GCG GCG CTG CCG GCC GTC GGC CAG ACC ACG CTG	531
	Val Asn Ala His Gly Ala Ala Leu Pro Ala Val Gly Gln Thr Thr Leu	
	160 165 170	
70	CCC GGT CAC ACC TAC GGT TCG CTG AGC AAC CTG GTC ACC GAG CTG ACC	579
	Pro Gly His Thr Tyr Gly Ser Leu Ser Asn Leu Val Thr Glu Leu Thr	
	175 180 185	

5	GCG GTC GTC TGG AAC GGC ACC ACC TAC GCA CTC GAG ACG TAC CAG CGC Ala Val Val Trp Asn Gly Thr Thr Tyr Ala Leu Glu Thr Tyr Gln Arg 190 195 200	627
10	AAC GAT CCT CGG ATC ACC CCA CTG CTC ACC AAC CTC GGG CGC TGC TTC Asn Asp Pro Arg Ile Thr Pro Leu Leu Thr Asn Leu Gly Arg Cys Phe 205 210 215	675
15	CTG ACC TCG GTG ACG ATG CAG GCC GGC CCC AAC TTC CGT CAG CGG TGC Leu Thr Ser Val Thr Met Gln Ala Gly Pro Asn Phe Arg Gln Arg Cys 220 225 230 235	723
20	CAG AGC TAC ACC GAC ATC CCG TGG CGG GAA CTG TTC GCG CCG AAG GGC Gln Ser Tyr Thr Asp Ile Pro Trp Arg Glu Leu Phe Ala Pro Lys Gly 240 245 250	771
25	GCC GAC GGC CGC ACG TTC GAG AAG TTC GTC GCG GAA TCG GGC GGC GCC Ala Asp Gly Arg Thr Phe Glu Lys Phe Val Ala Glu Ser Gly Gly Ala 255 260 265	819
30	GAG GCG ATC TGG TAC CCG TTC ACC GAG AAG CCG TGG ATG AAG GTG TGG Glu Ala Ile Trp Tyr Pro Phe Thr Glu Lys Pro Trp Met Lys Val Trp 270 275 280	867
35	ACG GTC TCG CCG ACC AAG CCG GAC TCG TCG AAC GAG GTC GGA AGC CTC Thr Val Ser Pro Thr Lys Pro Asp Ser Ser Asn Glu Val Gly Ser Leu 285 290 295	915
40	GGC TCG GCG GGC TCC CTC GTC GGC AAG CCT CCG CAG GCG CGT GAG GTC Gly Ser Ala Gly Ser Leu Val Gly Lys Pro Pro Gln Ala Arg Glu Val 300 305 310 315	963
45	TCC GGC CCG TAC AAC TAC ATC TTC TCC GAC AAC CTG CCG GAG CCC ATC Ser Gly Pro Tyr Asn Tyr Ile Phe Ser Asp Asn Leu Pro Glu Pro Ile 320 325 330	1011
50	ACC GAC ATG ATC GGC GCC ATC AAC GCC GGA AAC CCC GGA ATC GCA CCG Thr Asp Met Ile Gly Ala Ile Asn Ala Gly Asn Pro Gly Ile Ala Pro 335 340 345	1059
55	CTG TTC GGC CCG GCG ATG TAC GAG ATC ACC AAG CTC GGG CTG GCC GCG Leu Phe Gly Pro Ala Met Tyr Glu Ile Thr Lys Leu Gly Leu Ala Ala 350 355 360	1107
60	ACG AAT GCC AAC GAC ATC TGG GGC TGG TCG AAG GAC GTC CAG TTC TAC Thr Asn Ala Asn Asp Ile Trp Gly Trp Ser Lys Asp Val Gln Phe Tyr 365 370 375	1155
65	ATC AAG GCC ACG ACG TTG CGA CTC ACC GAG GGC GGC GGC GCC GTC GTC Ile Lys Ala Thr Thr Leu Arg Leu Thr Glu Gly Gly Gly Ala Val Val 380 385 390 395	1203
70	ACG AGC CGC GCC AAC ATC GCG ACC GTG ATC AAC GAC TTC ACC GAG TGG Thr Ser Arg Ala Asn Ile Ala Thr Val Ile Asn Asp Phe Thr Glu Trp 400 405 410	1251
75	TTC CAC GAG CGC ATC GAG TTC TAC CGC GCG AAG GGC GAG TTC CCG CTC Phe His Glu Arg Ile Glu Phe Tyr Arg Ala Lys Gly Glu Phe Pro Leu 415 420 425	1299
80	AAC GGT CCG GTC GAG ATC CGC TGC TGC GGG CTC GAT CAG GCA GCC GAC Asn Gly Pro Val Glu Ile Arg Cys Cys Gly Leu Asp Gln Ala Ala Asp 430 435 440	1347

5	GTC AAG GTG CCG TCG GTG GGC CCG CCG ACC ATC TCG GCG ACC CGT CCG Val Lys Val Pro Ser Val Gly Pro Pro Thr Ile Ser Ala Thr Arg Pro 445 450 455	1395
10	CGT CCG GAT CAT CCG GAC TGG GAC GTC GCG ATC TGG CTG AAC GTT CTC Arg Pro Asp His Pro Asp Trp Asp Val Ala Ile Trp Leu Asn Val Leu 460 465 470 475	1443
15	GGT GTT CCG GGC ACC CCC GGC ATG TTC GAG TTC TAC CGC GAG ATG GAG Gly Val Pro Gly Thr Pro Gly Met Phe Glu Phe Tyr Arg Glu Met Glu 480 485 490	1491
20	CAG TGG ATG CCG AGC CAC TAC AAC AAC GAC GAC GCC ACC TTC CGG CCC Gln Trp Met Arg Ser His Tyr Asn Asn Asp Asp Ala Thr Phe Arg Pro 495 500 505	1539
25	GAG TGG TCG AAG GGG TGG GCG TTC GGT CCC GAC CCG TAC ACC GAC AAC Glu Trp Ser Lys Gly Trp Ala Phe Gly Pro Asp Pro Tyr Thr Asp Asn 510 515 520	1587
30	GAC ATC GTC ACG AAC AAG ATG CGC GCC ACC TAC ATC GAA GGT GTC CCG Asp Ile Val Thr Asn Lys Met Arg Ala Thr Tyr Ile Glu Gly Val Pro 525 530 535	1635
35	ACG ACC GAG AAC TGG GAC ACC GCG CGC GCT CGG TAC AAC CAG ATC GAC Thr Thr Glu Asn Trp Asp Thr Ala Arg Ala Arg Tyr Asn Gln Ile Asp 540 545 550 555	1683
40	CCG CAT CGC GTG TTC ACC AAC GGA TTC ATG GAC AAG CTG CTT CCG Pro His Arg Val Phe Thr Asn Gly Phe Met Asp Lys Leu Leu Pro 560 565 570	1728

(2) INFORMATION ZU SEQ ID NO: 25:

35 (i) SEQUENZ CHARAKTERISTIKA:

(A) LANGE: 570 Aminosäuren

(B) ART: Aminosäure

(D) TOPOLOGIE: linear

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(ii) ART DES MOLEKÜLS: Protein

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 25:

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EP 0 698 102 B1

	Met	Ser	Asn	His	His	His	Gly	His	Ala	Ser	Thr	Gly	Pro	Val	Ala	Pro
	1				5					10					15	
5	Leu	Pro	Thr	Pro	Pro	Asn	Phe	Pro	Asn	Asp	Ile	Ala	Leu	Phe	Gln	Gln
				20					25					30		
	Ala	Tyr	Gln	Asn	Trp	Ser	Lys	Glu	Ile	Met	Leu	Asp	Ala	Thr	Trp	Val
			35					40					45			
10	Cys	Ser	Pro	Lys	Thr	Pro	Gln	Asp	Val	Val	Arg	Leu	Ala	Asn	Trp	Ala
		50					55					60				
	His	Glu	His	Asp	Tyr	Lys	Ile	Arg	Pro	Arg	Gly	Ala	Met	His	Gly	Trp
	65					70					75				80	
15	Thr	Pro	Leu	Thr	Val	Glu	Lys	Gly	Ala	Asn	Val	Glu	Lys	Val	Ile	Leu
					85					90					95	
20																
25																
30																
35																
40																
45																
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	Ala	Asp	Thr	Met	Thr	His	Leu	Asn	Gly	Ile	Thr	Val	Asn	Thr	Gly	Gly	
				100					105					110			
5	Pro	Val	Ala	Thr	Val	Thr	Ala	Gly	Ala	Gly	Ala	Ser	Ile	Glu	Ala	Ile	
			115					120					125				
	Val	Thr	Glu	Leu	Gln	Lys	His	Asp	Leu	Gly	Trp	Ala	Asn	Leu	Pro	Ala	
		130					135					140					
10	Pro	Gly	Val	Leu	Ser	Ile	Gly	Gly	Ala	Leu	Ala	Val	Asn	Ala	His	Gly	
	145					150					155					160	
	Ala	Ala	Leu	Pro	Ala	Val	Gly	Gln	Thr	Thr	Leu	Pro	Gly	His	Thr	Tyr	
					165					170					175		
15	Gly	Ser	Leu	Ser	Asn	Leu	Val	Thr	Glu	Leu	Thr	Ala	Val	Val	Trp	Asn	
			180						185					190			
	Gly	Thr	Thr	Tyr	Ala	Leu	Glu	Thr	Tyr	Gln	Arg	Asn	Asp	Pro	Arg	Ile	
			195					200					205				
20	Thr	Pro	Leu	Leu	Thr	Asn	Leu	Gly	Arg	Cys	Phe	Leu	Thr	Ser	Val	Thr	
		210					215						220				
	Met	Gln	Ala	Gly	Pro	Asn	Phe	Arg	Gln	Arg	Cys	Gln	Ser	Tyr	Thr	Asp	
25		225				230					235					240	
	Ile	Pro	Trp	Arg	Glu	Leu	Phe	Ala	Pro	Lys	Gly	Ala	Asp	Gly	Arg	Thr	
					245					250					255		
	Phe	Glu	Lys	Phe	Val	Ala	Glu	Ser	Gly	Gly	Ala	Glu	Ala	Ile	Trp	Tyr	
30				260					265					270			
	Pro	Phe	Thr	Glu	Lys	Pro	Trp	Met	Lys	Val	Trp	Thr	Val	Ser	Pro	Thr	
			275					280					285				
	Lys	Pro	Asp	Ser	Ser	Asn	Glu	Val	Gly	Ser	Leu	Gly	Ser	Ala	Gly	Ser	
35		290					295					300					
	Leu	Val	Gly	Lys	Pro	Pro	Gln	Ala	Arg	Glu	Val	Ser	Gly	Pro	Tyr	Asn	
		305				310					315					320	
	Tyr	Ile	Phe	Ser	Asp	Asn	Leu	Pro	Glu	Pro	Ile	Thr	Asp	Met	Ile	Gly	
40					325					330					335		
	Ala	Ile	Asn	Ala	Gly	Asn	Pro	Gly	Ile	Ala	Pro	Leu	Phe	Gly	Pro	Ala	
				340					345					350			
	Met	Tyr	Glu	Ile	Thr	Lys	Leu	Gly	Leu	Ala	Ala	Thr	Asn	Ala	Asn	Asp	
45			355					360					365				
	Ile	Trp	Gly	Trp	Ser	Lys	Asp	Val	Gln	Phe	Tyr	Ile	Lys	Ala	Thr	Thr	
		370					375					380					
	Leu	Arg	Leu	Thr	Glu	Gly	Gly	Gly	Ala	Val	Val	Thr	Ser	Arg	Ala	Asn	
50		385				390					395					400	
	Ile	Ala	Thr	Val	Ile	Asn	Asp	Phe	Thr	Glu	Trp	Phe	His	Glu	Arg	Ile	
				405						410				415			
55	Glu	Phe	Tyr	Arg	Ala	Lys	Gly	Glu	Phe	Pro	Leu	Asn	Gly	Pro	Val	Glu	
				420					425					430			

5 Ile Arg Cys Cys Gly Leu Asp Gln Ala Ala Asp Val Lys Val Pro Ser
 435 440 445
 Val Gly Pro Pro Thr Ile Ser Ala Thr Arg Pro Arg Pro Asp His Pro
 450 455 460
 10 Asp Trp Asp Val Ala Ile Trp Leu Asn Val Leu Gly Val Pro Gly Thr
 465 470 475 480
 Pro Gly Met Phe Glu Phe Tyr Arg Glu Met Glu Gln Trp Met Arg Ser
 485 490 495
 15 His Tyr Asn Asn Asp Asp Ala Thr Phe Arg Pro Glu Trp Ser Lys Gly
 500 505 510
 Trp Ala Phe Gly Pro Asp Pro Tyr Thr Asp Asn Asp Ile Val Thr Asn
 515 520 525
 20 Lys Met Arg Ala Thr Tyr Ile Glu Gly Val Pro Thr Thr Glu Asn Trp
 530 535 540
 Asp Thr Ala Arg Ala Arg Tyr Asn Gln Ile Asp Pro His Arg Val Phe
 545 550 555 560
 25 Thr Asn Gly Phe Met Asp Lys Leu Leu Pro
 565 570

(2) INFORMATION ZU SEQ ID NO: 26:

30 (i) SEQUENZ CHARAKTERISTIKA:

(A) LÄNGE: 1741 Basenpaare
 (B) ART: Nukleinsäure
 (C) STRANGFORM: Einzel
 35 (D) TOPOLOGIE: linear

(ix) MERKMALE:

(A) NAME/SCHLÜSSEL: CDS
 40 (B) LAGE: 20..1741

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 26:

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	GAATTTAAGG GGAACATCG	ATG AGT AAT ACG CGT AAA CGC AAG CGC CGT ACG	52
		Met Ser Asn Thr Arg Lys Arg Lys Arg Arg Thr	
		1 5 10	
5	CAT GCC TCG ACC GGG CCG GTC GCG CCG CTT CCG ACG CCG CCG AAC TTC		100
	His Ala Ser Thr Gly Pro Val Ala Pro Leu Pro Thr Pro Pro Asn Phe		
	15 20 25		
10	CCG AAC GAC ATC GCG CTG TTC CAG CAG GCG TAC CAG AAC TGG TCC AAG		148
	Pro Asn Asp Ile Ala Leu Phe Gln Gln Ala Tyr Gln Asn Trp Ser Lys		
	30 35 40		
15	GAG ATC ATG CTG GAC GCC ACT TGG GTC TGC TCG CCC AAG ACG CCG CAG		196
	Glu Ile Met Leu Asp Ala Thr Trp Val Cys Ser Pro Lys Thr Pro Gln		
	45 50 55		
20	GAT GTC GTT CGC CTT GCC AAC TGG GCG CAC GAG CAC GAC TAC AAG ATC		244
	Asp Val Val Arg Leu Ala Asn Trp Ala His Glu His Asp Tyr Lys Ile		
	60 65 70 75		
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	CGC	CCG	CGC	GGC	GCG	ATG	CAC	GGC	TGG	ACC	CCG	CTC	ACC	GTG	GAG	AAG	292
	Arg	Pro	Arg	Gly	Ala	Met	His	Gly	Trp	Thr	Pro	Leu	Thr	Val	Glu	Lys	
				80					85						90		
5	GGG	GCC	AAC	GTC	GAG	AAG	GTG	ATC	CTC	GCC	GAC	ACG	ATG	ACG	CAT	CTG	340
	Gly	Ala	Asn	Val	Glu	Lys	Val	Ile	Leu	Ala	Asp	Thr	Met	Thr	His	Leu	
				95				100					105				
10	AAC	GGC	ATC	ACG	GTG	AAC	ACG	GGC	GGC	CCC	GTG	GCT	ACC	GTC	ACC	GCC	388
	Asn	Gly	Ile	Thr	Val	Asn	Thr	Gly	Gly	Pro	Val	Ala	Thr	Val	Thr	Ala	
			110					115					120				
	GGT	GCC	GGC	GCC	AGC	ATC	GAG	GCG	ATC	GTC	ACC	GAA	CTG	CAG	AAG	CAC	436
	Gly	Ala	Gly	Ala	Ser	Ile	Glu	Ala	Ile	Val	Thr	Glu	Leu	Gln	Lys	His	
			125				130					135					
15	GAC	CTC	GGC	TGG	GCC	AAC	CTG	CCC	GCT	CCG	GGT	GTG	CTG	TCG	ATC	GGT	484
	Asp	Leu	Gly	Trp	Ala	Asn	Leu	Pro	Ala	Pro	Gly	Val	Leu	Ser	Ile	Gly	
						145					150					155	
20	GGC	GCC	CTT	GCG	GTC	AAC	GCG	CAC	GGT	GCG	GCG	CTG	CCG	GCC	GTC	GGC	532
	Gly	Ala	Leu	Ala	Val	Asn	Ala	His	Gly	Ala	Ala	Leu	Pro	Ala	Val	Gly	
					160					165					170		
	CAG	ACC	ACG	CTG	CCC	GGT	CAC	ACC	TAC	GGT	TCG	CTG	AGC	AAC	CTG	GTC	580
	Gln	Thr	Thr	Leu	Pro	Gly	His	Thr	Tyr	Gly	Ser	Leu	Ser	Asn	Leu	Val	
				175					180					185			
25	ACC	GAG	CTG	ACC	GCG	GTC	GTC	TGG	AAC	GGC	ACC	ACC	TAC	GCA	CTC	GAG	628
	Thr	Glu	Leu	Thr	Ala	Val	Val	Trp	Asn	Gly	Thr	Thr	Tyr	Ala	Leu	Glu	
				190				195					200				
30	ACG	TAC	CAG	CGC	AAC	GAT	CCT	CGG	ATC	ACC	CCA	CTG	CTC	ACC	AAC	CTC	676
	Thr	Tyr	Gln	Arg	Asn	Asp	Pro	Arg	Ile	Thr	Pro	Leu	Leu	Thr	Asn	Leu	
		205					210					215					
	GGG	CGC	TGC	TTC	CTG	ACC	TCG	GTG	ACG	ATG	CAG	GCC	GGC	CCC	AAC	TTC	724
	Gly	Arg	Cys	Phe	Leu	Thr	Ser	Val	Thr	Met	Gln	Ala	Gly	Pro	Asn	Phe	
		220				225					230					235	
35	CGT	CAG	CGG	TGC	CAG	AGC	TAC	ACC	GAC	ATC	CCG	TGG	CGG	GAA	CTG	TTC	772
	Arg	Gln	Arg	Cys	Gln	Ser	Tyr	Thr	Asp	Ile	Pro	Trp	Arg	Glu	Leu	Phe	
					240				245						250		
40	GCG	CCG	AAG	GGC	GCC	GAC	GGC	CGC	ACG	TTC	GAG	AAG	TTC	GTC	GCG	GAA	820
	Ala	Pro	Lys	Gly	Ala	Asp	Gly	Arg	Thr	Phe	Glu	Lys	Phe	Val	Ala	Glu	
				255					260					265			
	TCG	GGC	GGC	GCC	GAG	GCG	ATC	TGG	TAC	CCG	TTC	ACC	GAG	AAG	CCG	TGG	868
	Ser	Gly	Gly	Ala	Glu	Ala	Ile	Trp	Tyr	Pro	Phe	Thr	Glu	Lys	Pro	Trp	
			270				275						280				
	ATG	AAG	GTG	TGG	ACG	GTC	TCG	CCG	ACC	AAG	CCG	GAC	TCG	TCG	AAC	GAG	916
	Met	Lys	Val	Trp	Thr	Val	Ser	Pro	Thr	Lys	Pro	Asp	Ser	Ser	Asn	Glu	
			285				290					295					
50	GTC	GGA	AGC	CTC	GGC	TCG	GCG	GGC	TCC	CTC	GTC	GGC	AAG	CCT	CCG	CAG	964
	Val	Gly	Ser	Leu	Gly	Ser	Ala	Gly	Ser	Leu	Val	Gly	Lys	Pro	Pro	Gln	
						305					310					315	
55	GCG	CGT	GAG	GTC	TCC	GGC	CCG	TAC	AAC	TAC	ATC	TTC	TCC	GAC	AAC	CTG	1012
	Ala	Arg	Glu	Val	Ser	Gly	Pro	Tyr	Asn	Tyr	Ile	Phe	Ser	Asp	Asn	Leu	
					320					325					330		

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	CCG GAG CCC ATC ACC GAC ATG ATC GGC GCC ATC AAC GCC GGA AAC CCC	1060
	Pro Glu Pro Ile Thr Asp Met Ile Gly Ala Ile Asn Ala Gly Asn Pro	
	335 340 345	
5	GGA ATC GCA CCG CTG TTC GGC CCG GCG ATG TAC GAG ATC ACC AAG CTC	1108
	Gly Ile Ala Pro Leu Phe Gly Pro Ala Met Tyr Glu Ile Thr Lys Leu	
	350 355 360	
10	GGG CTG GCC GCG ACG AAT GCC AAC GAC ATC TGG GGC TGG TCG AAG GAC	1156
	Gly Leu Ala Ala Thr Asn Ala Asn Asp Ile Trp Gly Trp Ser Lys Asp	
	365 370 375	
15	GTC CAG TTC TAC ATC AAG GCC ACG ACG TTG CGA CTC ACC GAG GGC GGC	1204
	Val Gln Phe Tyr Ile Lys Ala Thr Thr Leu Arg Leu Thr Glu Gly Gly	
	380 385 390 395	
20	GGC GCC GTC GTC ACG AGC CGC GCC AAC ATC GCG ACC GTG ATC AAC GAC	1252
	Gly Ala Val Val Thr Ser Arg Ala Asn Ile Ala Thr Val Ile Asn Asp	
	400 405 410	
25	TTC ACC GAG TGG TTC CAC GAG CGC ATC GAG TTC TAC CGC GCG AAG GGC	1300
	Phe Thr Glu Trp Phe His Glu Arg Ile Glu Phe Tyr Arg Ala Lys Gly	
	415 420 425	
30	GAG TTC CCG CTC AAC GGT CCG GTC GAG ATC CGC TGC TGC GGG CTC GAT	1348
	Glu Phe Pro Leu Asn Gly Pro Val Glu Ile Arg Cys Cys Gly Leu Asp	
	430 435 440	
35	CAG GCA GCC GAC GTC AAG GTG CCG TCG GTG GGC CCG CCG ACC ATC TCG	1396
	Gln Ala Ala Asp Val Lys Val Pro Ser Val Gly Pro Pro Thr Ile Ser	
	445 450 455	
40	GCG ACC CGT CCG CGT CCG GAT CAT CCG GAC TGG GAC GTC GCG ATC TGG	1444
	Ala Thr Arg Pro Arg Pro Asp His Pro Asp Trp Asp Val Ala Ile Trp	
	460 465 470 475	
45	CTG AAC GTT CTC GGT GTT CCG GGC ACC CCC GGC ATG TTC GAG TTC TAC	1492
	Leu Asn Val Leu Gly Val Pro Gly Thr Pro Gly Met Phe Glu Phe Tyr	
	480 485 490	
50	CGC GAG ATG GAG CAG TGG ATG CGG AGC CAC TAC AAC AAC GAC GAC GCC	1540
	Arg Glu Met Glu Gln Trp Met Arg Ser His Tyr Asn Asn Asp Asp Ala	
	495 500 505	
55	ACC TTC CGG CCC GAG TGG TCG AAG GGG TGG GCG TTC GGT CCC GAC CCG	1588
	Thr Phe Arg Pro Glu Trp Ser Lys Gly Trp Ala Phe Gly Pro Asp Pro	
	510 515 520	
60	TAC ACC GAC AAC GAC ATC GTC ACG AAC AAG ATG CGC GCC ACC TAC ATC	1636
	Tyr Thr Asp Asn Asp Ile Val Thr Asn Lys Met Arg Ala Thr Tyr Ile	
	525 530 535	
65	GAA GGT GTC CCG ACG ACC GAG AAC TGG GAC ACC GCG CGC GCT CGG TAC	1684
	Glu Gly Val Pro Thr Thr Glu Asn Trp Asp Thr Ala Arg Ala Arg Tyr	
	540 545 550 555	
70	AAC CAG ATC GAC CCG CAT CGC GTG TTC ACC AAC GGA TTC ATG GAC AAG	1732
	Asn Gln Ile Asp Pro His Arg Val Phe Thr Asn Gly Phe Met Asp Lys	
	560 565 570	
75	CTG CTT CCG	1741
	Leu Leu Pro	

(2) INFORMATION ZU SEQ ID NO: 27:

(i) SEQUENZ CHARAKTERISTIKA:

5 (A) LANGE: 574 Aminosäuren
(B) ART: Aminosäure
(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Protein
10 (xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 27:

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Met Ser Asn Thr Arg Lys Arg Lys Arg Arg Thr His Ala Ser Thr Gly
 1 5 10 15
 5 Pro Val Ala Pro Leu Pro Thr Pro Pro Asn Phe Pro Asn Asp Ile Ala
 20 25 30
 Leu Phe Gln Gln Ala Tyr Gln Asn Trp Ser Lys Glu Ile Met Leu Asp
 35 40 45
 10 Ala Thr Trp Val Cys Ser Pro Lys Thr Pro Gln Asp Val Val Arg Leu
 50 55 60
 Ala Asn Trp Ala His Glu His Asp Tyr Lys Ile Arg Pro Arg Gly Ala
 65 70 75 80
 15 Met His Gly Trp Thr Pro Leu Thr Val Glu Lys Gly Ala Asn Val Glu
 85 90 95
 Lys Val Ile Leu Ala Asp Thr Met Thr His Leu Asn Gly Ile Thr Val
 100 105 110
 20 Asn Thr Gly Gly Pro Val Ala Thr Val Thr Ala Gly Ala Gly Ala Ser
 115 120 125
 Ile Glu Ala Ile Val Thr Glu Leu Gln Lys His Asp Leu Gly Trp Ala
 130 135 140
 25 Asn Leu Pro Ala Pro Gly Val Leu Ser Ile Gly Gly Ala Leu Ala Val
 145 150 155 160
 Asn Ala His Gly Ala Ala Leu Pro Ala Val Gly Gln Thr Thr Leu Pro
 165 170 175
 Gly His Thr Tyr Gly Ser Leu Ser Asn Leu Val Thr Glu Leu Thr Ala
 180 185 190
 30 Val Val Trp Asn Gly Thr Thr Tyr Ala Leu Glu Thr Tyr Gln Arg Asn
 195 200 205
 Asp Pro Arg Ile Thr Pro Leu Leu Thr Asn Leu Gly Arg Cys Phe Leu
 210 215 220
 40 Thr Ser Val Thr Met Gln Ala Gly Pro Asn Phe Arg Gln Arg Cys Gln
 225 230 235 240
 Ser Tyr Thr Asp Ile Pro Trp Arg Glu Leu Phe Ala Pro Lys Gly Ala
 245 250 255
 45 Asp Gly Arg Thr Phe Glu Lys Phe Val Ala Glu Ser Gly Gly Ala Glu
 260 265 270

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Ala Ile Trp Tyr Pro Phe Thr Glu Lys Pro Trp Met Lys Val Trp Thr
 275 280 285
 5 Val Ser Pro Thr Lys Pro Asp Ser Ser Asn Glu Val Gly Ser Leu Gly
 290 295 300
 Ser Ala Gly Ser Leu Val Gly Lys Pro Pro Gln Ala Arg Glu Val Ser
 305 310 315 320
 10 Gly Pro Tyr Asn Tyr Ile Phe Ser Asp Asn Leu Pro Glu Pro Ile Thr
 325 330 335
 Asp Met Ile Gly Ala Ile Asn Ala Gly Asn Pro Gly Ile Ala Pro Leu
 340 345 350
 15 Phe Gly Pro Ala Met Tyr Glu Ile Thr Lys Leu Gly Leu Ala Ala Thr
 355 360 365
 Asn Ala Asn Asp Ile Trp Gly Trp Ser Lys Asp Val Gln Phe Tyr Ile
 370 375 380
 20 Lys Ala Thr Thr Leu Arg Leu Thr Glu Gly Gly Gly Ala Val Val Thr
 385 390 395 400
 Ser Arg Ala Asn Ile Ala Thr Val Ile Asn Asp Phe Thr Glu Trp Phe
 405 410 415
 25 His Glu Arg Ile Glu Phe Tyr Arg Ala Lys Gly Glu Phe Pro Leu Asn
 420 425 430
 Gly Pro Val Glu Ile Arg Cys Cys Gly Leu Asp Gln Ala Ala Asp Val
 435 440 445
 30 Lys Val Pro Ser Val Gly Pro Pro Thr Ile Ser Ala Thr Arg Pro Arg
 450 455 460
 Pro Asp His Pro Asp Trp Asp Val Ala Ile Trp Leu Asn Val Leu Gly
 465 470 475 480
 Val Pro Gly Thr Pro Gly Met Phe Glu Phe Tyr Arg Glu Met Glu Gln
 485 490 495
 40 Trp Met Arg Ser His Tyr Asn Asn Asp Asp Ala Thr Phe Arg Pro Glu
 500 505 510
 Trp Ser Lys Gly Trp Ala Phe Gly Pro Asp Pro Tyr Thr Asp Asn Asp
 515 520 525
 45 Ile Val Thr Asn Lys Met Arg Ala Thr Tyr Ile Glu Gly Val Pro Thr
 530 535 540
 Thr Glu Asn Trp Asp Thr Ala Arg Ala Arg Tyr Asn Gln Ile Asp Pro
 545 550 555 560
 50 His Arg Val Phe Thr Asn Gly Phe Met Asp Lys Leu Leu Pro
 565 570

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(2) INFORMATION ZU SEQ ID NO: 28:

(i) SEQUENZ CHARAKTERISTIKA:

EP 0 698 102 B1

(A) LÄNGE: 1731 Basenpaare
(B) ART: Nukleinsäure
(C) STRANGFORM: Einzel
(D) TOPOLOGIE: linear

(ix) MERKMALE:

(A) NAME/SCHLÜSSEL: CDS
(B) LAGE: 25..1731

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 28:

15	GAATTCACAC AGGAAACAGA ATTC ATG GTT ATG CAC CAT GGG CAT GCC TCG	51
	Met Val Met His His Gly His Ala Ser	
	1 5	
20	ACC GGG CCG GTC GCG CCG CTT CCG ACG CCG CCG AAC TTC CCG AAC GAC	99
	Thr Gly Pro Val Ala Pro Leu Pro Thr Pro Pro Asn Phe Pro Asn Asp	
	10 15 20 25	
25	ATC GCG CTG TTC CAG CAG GCG TAC CAG AAC TGG TCC AAG GAG ATC ATG	147
	Ile Ala Leu Phe Gln Gln Ala Tyr Gln Asn Trp Ser Lys Glu Ile Met	
	30 35 40	
30	CTG GAC GCC ACT TGG GTC TGC TCG CCC AAG ACG CCG CAG GAT GTC GTT	195
	Leu Asp Ala Thr Trp Val Cys Ser Pro Lys Thr Pro Gln Asp Val Val	
	45 50 55	
35	CGC CTT GCC AAC TGG GCG CAC GAG CAC GAC TAC AAG ATC CGC CCG CGC	243
	Arg Leu Ala Asn Trp Ala His Glu His Asp Tyr Lys Ile Arg Pro Arg	
	60 65 70	
40	GGC GCG ATG CAC GGC TGG ACC CCG CTC ACC GTG GAG AAG GGG GCC AAC	291
	Gly Ala Met His Gly Trp Thr Pro Leu Thr Val Glu Lys Gly Ala Asn	
	75 80 85	
45	GTC GAG AAG GTG ATC CTC GCC GAC ACG ATG ACG CAT CTG AAC GGC ATC	339
	Val Glu Lys Val Ile Leu Ala Asp Thr Met Thr His Leu Asn Gly Ile	
	90 95 100 105	
50	ACG GTG AAC ACG GGC GGC CCC GTG GCT ACC GTC ACC GCC GGT GCC GGC	387
	Thr Val Asn Thr Gly Gly Pro Val Ala Thr Val Thr Ala Gly Ala Gly	
	110 115 120	
55	GCC AGC ATC GAG GCG ATC GTC ACC GAA CTG CAG AAG CAC GAC CTC GGC	435
	Ala Ser Ile Glu Ala Ile Val Thr Glu Leu Gln Lys His Asp Leu Gly	
	125 130 135	
60	TGG GCC AAC CTG CCC GCT CCG GGT GTG CTG TCG ATC GGT GGC GCC CTT	483
	Trp Ala Asn Leu Pro Ala Pro Gly Val Leu Ser Ile Gly Gly Ala Leu	
	140 145 150	
65	GCG GTC AAC GCG CAC GGT GCG GCG CTG CCG GCC GTC GGC CAG ACC ACG	531
	Ala Val Asn Ala His Gly Ala Ala Leu Pro Ala Val Gly Gln Thr Thr	
	155 160 165	
70	CTG CCC GGT CAC ACC TAC GGT TCG CTG AGC AAC CTG GTC ACC GAG CTG	579
	Leu Pro Gly His Thr Tyr Gly Ser Leu Ser Asn Leu Val Thr Glu Leu	
	170 175 180 185	

	ACC	GCG	GTC	GTC	TGG	AAC	GGC	ACC	ACC	TAC	GCA	CTC	GAG	ACG	TAC	CAG	627
	Thr	Ala	Val	Val	Trp	Asn	Gly	Thr	Thr	Tyr	Ala	Leu	Glu	Thr	Tyr	Gln	
					190					195					200		
5	CGC	AAC	GAT	CCT	CGG	ATC	ACC	CCA	CTG	CTC	ACC	AAC	CTC	GGG	CGC	TGC	675
	Arg	Asn	Asp	Pro	Arg	Ile	Thr	Pro	Leu	Leu	Thr	Asn	Leu	Gly	Arg	Cys	
				205					210					215			
10	TTC	CTG	ACC	TCG	GTG	ACG	ATG	CAG	GCC	GGC	CCC	AAC	TTC	CGT	CAG	CGG	723
	Phe	Leu	Thr	Ser	Val	Thr	Met	Gln	Ala	Gly	Pro	Asn	Phe	Arg	Gln	Arg	
			220					225					230				
15	TGC	CAG	AGC	TAC	ACC	GAC	ATC	CCG	TGG	CGG	GAA	CTG	TTC	GCG	CCG	AAG	771
	Cys	Gln	Ser	Tyr	Thr	Asp	Ile	Pro	Trp	Arg	Glu	Leu	Phe	Ala	Pro	Lys	
		235					240					245					
	GGC	GCC	GAC	GGC	CGC	ACG	TTC	GAG	AAG	TTC	GTC	GCG	GAA	TCG	GGC	GGC	819
	Gly	Ala	Asp	Gly	Arg	Thr	Phe	Glu	Lys	Phe	Val	Ala	Glu	Ser	Gly	Gly	
	250					255					260					265	
20	GCC	GAG	GCG	ATC	TGG	TAC	CCG	TTC	ACC	GAG	AAG	CCG	TGG	ATG	AAG	GTG	867
	Ala	Glu	Ala	Ile	Trp	Tyr	Pro	Phe	Thr	Glu	Lys	Pro	Trp	Met	Lys	Val	
					270					275					280		
25	TGG	ACG	GTC	TCG	CCG	ACC	AAG	CCG	GAC	TCG	TCG	AAC	GAG	GTC	GGA	AGC	915
	Trp	Thr	Val	Ser	Pro	Thr	Lys	Pro	Asp	Ser	Ser	Asn	Glu	Val	Gly	Ser	
				285				290						295			
	CTC	GGC	TCG	GCG	GGC	TCC	CTC	GTC	GGC	AAG	CCT	CCG	CAG	GCG	CGT	GAG	963
	Leu	Gly	Ser	Ala	Gly	Ser	Leu	Val	Gly	Lys	Pro	Pro	Gln	Ala	Arg	Glu	
			300					305					310				
30	GTC	TCC	GGC	CCG	TAC	AAC	TAC	ATC	TTC	TCC	GAC	AAC	CTG	CCG	GAG	CCC	1011
	Val	Ser	Gly	Pro	Tyr	Asn	Tyr	Ile	Phe	Ser	Asp	Asn	Leu	Pro	Glu	Pro	
		315					320					325					
35	ATC	ACC	GAC	ATG	ATC	GGC	GCC	ATC	AAC	GCC	GGA	AAC	CCC	GGA	ATC	GCA	1059
	Ile	Thr	Asp	Met	Ile	Gly	Ala	Ile	Asn	Ala	Gly	Asn	Pro	Gly	Ile	Ala	
	330				335						340				345		
	CCG	CTG	TTC	GGC	CCG	GCG	ATG	TAC	GAG	ATC	ACC	AAG	CTC	GGG	CTG	GCC	1107
	Pro	Leu	Phe	Gly	Pro	Ala	Met	Tyr	Glu	Ile	Thr	Lys	Leu	Gly	Leu	Ala	
					350					355					360		
40	GCG	ACG	AAT	GCC	AAC	GAC	ATC	TGG	GGC	TGG	TCG	AAG	GAC	GTC	CAG	TTC	1155
	Ala	Thr	Asn	Ala	Asn	Asp	Ile	Trp	Gly	Trp	Ser	Lys	Asp	Val	Gln	Phe	
				365				370						375			
45	TAC	ATC	AAG	GCC	ACG	ACG	TTG	CGA	CTC	ACC	GAG	GGC	GGC	GGC	GCC	GTC	1203
	Tyr	Ile	Lys	Ala	Thr	Thr	Leu	Arg	Leu	Thr	Glu	Gly	Gly	Gly	Ala	Val	
			380				385						390				
	GTC	ACG	AGC	CGC	GCC	AAC	ATC	GCG	ACC	GTG	ATC	AAC	GAC	TTC	ACC	GAG	1251
	Val	Thr	Ser	Arg	Ala	Asn	Ile	Ala	Thr	Val	Ile	Asn	Asp	Phe	Thr	Glu	
		395					400					405					
50	TGG	TTC	CAC	GAG	CGC	ATC	GAG	TTC	TAC	CGC	GCG	AAG	GGC	GAG	TTC	CCG	1299
	Trp	Phe	His	Glu	Arg	Ile	Glu	Phe	Tyr	Arg	Ala	Lys	Gly	Glu	Phe	Pro	
	410					415					420					425	
55	CTC	AAC	GGT	CCG	GTC	GAG	ATC	CGC	TGC	TGC	GGG	CTC	GAT	CAG	GCA	GCC	1347
	Leu	Asn	Gly	Pro	Val	Glu	Ile	Arg	Cys	Cys	Gly	Leu	Asp	Gln	Ala	Ala	
					430					435					440		

5	GAC GTC AAG GTG CCG TCG GTG GGC CCG CCG ACC ATC TCG GCG ACC CGT Asp Val Lys Val Pro Ser Val Gly Pro Pro Thr Ile Ser Ala Thr Arg 445 450 455	1395
10	CCG CGT CCG GAT CAT CCG GAC TGG GAC GTC GCG ATC TGG CTG AAC GTT Pro Arg Pro Asp His Pro Asp Trp Asp Val Ala Ile Trp Leu Asn Val 460 465 470	1443
15	CTC GGT GTT CCG GGC ACC CCC GGC ATG TTC GAG TTC TAC CGC GAG ATG Leu Gly Val Pro Gly Thr Pro Gly Met Phe Glu Phe Tyr Arg Glu Met 475 480 485	1491
20	GAG CAG TGG ATG CCG AGC CAC TAC AAC AAC GAC GAC GCC ACC TTC CGG Glu Gln Trp Met Arg Ser His Tyr Asn Asn Asp Asp Ala Thr Phe Arg 490 495 500 505	1539
25	CCC GAG TGG TCG AAG GGG TGG GCG TTC GGT CCC GAC CCG TAC ACC GAC Pro Glu Trp Ser Lys Gly Trp Ala Phe Gly Pro Asp Pro Tyr Thr Asp 510 515 520	1587
30	AAC GAC ATC GTC ACG AAC AAG ATG CGC GCC ACC TAC ATC GAA GGT GTC Asn Asp Ile Val Thr Asn Lys Met Arg Ala Thr Tyr Ile Glu Gly Val 525 530 535	1635
35	CCG ACG ACC GAG AAC TGG GAC ACC GCG CGC GCT CGG TAC AAC CAG ATC Pro Thr Thr Glu Asn Trp Asp Thr Ala Arg Ala Arg Tyr Asn Gln Ile 540 545 550	1683
40	GAC CCG CAT CGC GTG TTC ACC AAC GGA TTC ATG GAC AAG CTG CTT CCG Asp Pro His Arg Val Phe Thr Asn Gly Phe Met Asp Lys Leu Leu Pro 555 560 565	1731

(2) INFORMATION ZU SEQ ID NO: 29:

(i) SEQUENZ CHARAKTERISTIKA:

(A) LANGE: 569 Aminosäuren

(B) ART: Aminosäure

(D) TOPOLOGIE: linear

(ii) ART DES MOLEKÜLS: Protein

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 29:

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	Met	Val	Met	His	His	Gly	His	Ala	Ser	Thr	Gly	Pro	Val	Ala	Pro	Leu
	1				5					10					15	
5	Pro	Thr	Pro	Pro	Asn	Phe	Pro	Asn	Asp	Ile	Ala	Leu	Phe	Gln	Gln	Ala
				20					25					30		
	Tyr	Gln	Asn	Trp	Ser	Lys	Glu	Ile	Met	Leu	Asp	Ala	Thr	Trp	Val	Cys
			35					40					45			
10	Ser	Pro	Lys	Thr	Pro	Gln	Asp	Val	Val	Arg	Leu	Ala	Asn	Trp	Ala	His
		50					55					60				
	Glu	His	Asp	Tyr	Lys	Ile	Arg	Pro	Arg	Gly	Ala	Met	His	Gly	Trp	Thr
15	65					70					75				80	
	Pro	Leu	Thr	Val	Glu	Lys	Gly	Ala	Asn	Val	Glu	Lys	Val	Ile	Leu	Ala
					85					90					95	

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Asp Thr Met Thr His Leu Asn Gly Ile Thr Val Asn Thr Gly Gly Pro
 100 105 110
 5 Val Ala Thr Val Thr Ala Gly Ala Gly Ala Ser Ile Glu Ala Ile Val
 115 120 125
 Thr Glu Leu Gln Lys His Asp Leu Gly Trp Ala Asn Leu Pro Ala Pro
 130 135 140
 10 Gly Val Leu Ser Ile Gly Gly Ala Leu Ala Val Asn Ala His Gly Ala
 145 150 155 160
 Ala Leu Pro Ala Val Gly Gln Thr Thr Leu Pro Gly His Thr Tyr Gly
 165 170 175
 15 Ser Leu Ser Asn Leu Val Thr Glu Leu Thr Ala Val Val Trp Asn Gly
 180 185 190
 Thr Thr Tyr Ala Leu Glu Thr Tyr Gln Arg Asn Asp Pro Arg Ile Thr
 195 200 205
 20 Pro Leu Leu Thr Asn Leu Gly Arg Cys Phe Leu Thr Ser Val Thr Met
 210 215 220
 Gln Ala Gly Pro Asn Phe Arg Gln Arg Cys Gln Ser Tyr Thr Asp Ile
 225 230 235 240
 25 Pro Trp Arg Glu Leu Phe Ala Pro Lys Gly Ala Asp Gly Arg Thr Phe
 245 250 255
 Glu Lys Phe Val Ala Glu Ser Gly Gly Ala Glu Ala Ile Trp Tyr Pro
 260 265 270
 30 Phe Thr Glu Lys Pro Trp Met Lys Val Trp Thr Val Ser Pro Thr Lys
 275 280 285
 Pro Asp Ser Ser Asn Glu Val Gly Ser Leu Gly Ser Ala Gly Ser Leu
 290 295 300
 35 Val Gly Lys Pro Pro Gln Ala Arg Glu Val Ser Gly Pro Tyr Asn Tyr
 305 310 315 320
 Ile Phe Ser Asp Asn Leu Pro Glu Pro Ile Thr Asp Met Ile Gly Ala
 325 330 335
 40 Ile Asn Ala Gly Asn Pro Gly Ile Ala Pro Leu Phe Gly Pro Ala Met
 340 345 350
 Tyr Glu Ile Thr Lys Leu Gly Leu Ala Ala Thr Asn Ala Asn Asp Ile
 355 360 365
 45 Trp Gly Trp Ser Lys Asp Val Gln Phe Tyr Ile Lys Ala Thr Thr Leu
 370 375 380
 Arg Leu Thr Glu Gly Gly Gly Ala Val Val Thr Ser Arg Ala Asn Ile
 385 390 395 400
 50 Ala Thr Val Ile Asn Asp Phe Thr Glu Trp Phe His Glu Arg Ile Glu
 405 410 415
 55 Phe Tyr Arg Ala Lys Gly Glu Phe Pro Leu Asn Gly Pro Val Glu Ile
 420 425 430

5 Arg Cys Cys Gly Leu Asp Gln Ala Ala Asp Val Lys Val Pro Ser Val
 435 440 445
 Gly Pro Pro Thr Ile Ser Ala Thr Arg Pro Arg Pro Asp His Pro Asp
 450 455 460
 10 Trp Asp Val Ala Ile Trp Leu Asn Val Leu Gly Val Pro Gly Thr Pro
 465 470 475 480
 Gly Met Phe Glu Phe Tyr Arg Glu Met Glu Gln Trp Met Arg Ser His
 485 490 495
 15 Tyr Asn Asn Asp Asp Ala Thr Phe Arg Pro Glu Trp Ser Lys Gly Trp
 500 505 510
 Ala Phe Gly Pro Asp Pro Tyr Thr Asp Asn Asp Ile Val Thr Asn Lys
 515 520 525
 20 Met Arg Ala Thr Tyr Ile Glu Gly Val Pro Thr Thr Glu Asn Trp Asp
 530 535 540
 Thr Ala Arg Ala Arg Tyr Asn Gln Ile Asp Pro His Arg Val Phe Thr
 545 550 555 560
 25 Asn Gly Phe Met Asp Lys Leu Leu Pro
 565

(2) INFORMATION ZU SEQ ID NO: 30:

30 (i) SEQUENZ CHARAKTERISTIKA:

- 35 (A) LÄNGE: 36 Basenpaare
 (B) ART: Nukleinsäure
 (C) STRANGFORM: Einzel
 (D) TOPOLOGIE: linear

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 30:

40 TCGCATGCCT CGACGGGCCC GGTGGCGCCG CTTCGG

36

(2) INFORMATION ZU SEQ ID NO: 31:

45 (i) SEQUENZ CHARAKTERISTIKA:

- 50 (A) LÄNGE: 25 Basenpaare
 (B) ART: Nukleinsäure
 (C) STRANGFORM: Einzel
 (D) TOPOLOGIE: linear

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 31:

55 CGTGCTTCTG CAGTTCGGTG ACGAT

25

(2) INFORMATION ZU SEQ ID NO: 32:

(i) SEQUENZ CHARAKTERISTIKA:

- (A) LANGE: 39 Basenpaare
(B) ART: Nukleinsäure-
(C) STRANGFORM: Einzel
(D) TOPOLOGIE: linear

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 32:

TCCCATGGCA CACAGGAAAC ATCGATGACC ATGATTACG

39

(2) INFORMATION ZU SEQ ID NO: 33:

(i) SEQUENZ CHARAKTERISTIKA:

- (A) LÄNGE: 25 Basenpaare
(B) ART: Nukleinsäure
(C) STRANGFORM: Einzel
(D) TOPOLOGIE: linear

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 33:

CGTGCTTCTG CAGTTCGGTG ACGAT

25

(2) INFORMATION ZU SEQ ID NO: 34:

(i) SEQUENZ CHARAKTERISTIKA:

- (A) LÄNGE: 18 Basenpaare
(B) ART: Nukleinsäure
(C) STRANGFORM: Einzel
(D) TOPOLOGIE: linear

(xi) SEQUENZBESCHREIBUNG: SEQ ID NO: 34:

CGATGCACCA TGGGCATG

18

Patentansprüche

1. Aktive Cholesterinoxidase, **dadurch gekennzeichnet, daß** sie die in SEQ ID NO 2 gezeigte Aminosäuresequenz aufweist.
2. DNA, welche für ein Peptid mit Cholesterinoxidase-Aktivität kodiert mit der in SEQ ID NO 1 gezeigten DNA-Sequenz oder der dazu komplementären DNA-Sequenz.
3. Verfahren zur Herstellung einer rekombinanten Cholesterinoxidase durch Transformation einer geeigneten Wirtszelle mit einer DNA gemäß Anspruch 2, welche in einem geeigneten Expressionssystem kloniert vorliegt, Kultivierung der transformierten Wirtszellen und Isolierung der exprimierten Cholesterinoxidase aus dem Zytoplasma der transformierten Zellen.
4. Verfahren gemäß Anspruch 3, **dadurch gekennzeichnet, daß** die verwendeten DNA am 5'-Ende eine der in SEQ

ID NO 6, 8, 10, 12, 14 oder 16 gezeigten Sequenzen aufweist.

5. DNA gemäß Anspruch 2, **dadurch gekennzeichnet, daß** sie am 5'-Ende eine der in SEQ ID NO 6, 8, 10, 12, 14 oder 16 gezeigten Sequenzen aufweist.
6. DNA gemäß Anspruch 5, **dadurch gekennzeichnet, daß** sie eine der in SEQ ID NO 18, 20, 22, 24, 26 oder 28 gezeigten Sequenzen aufweist.
7. Rekombinante Cholesterinoxidase, **dadurch gekennzeichnet, daß** sie von einer DNA gemäß Anspruch 2 kodiert wird und am N-terminalen Ende eine der in SEQ ID NO 7, 9, 11, 13, 15 oder 17 gezeigten Sequenzen aufweist.
8. Rekombinante Cholesterinoxidase gemäß Anspruch 7, **dadurch gekennzeichnet, daß** sie eine der in SEQ ID NO 21, 23, 25, 27 und 29 gezeigten Sequenzen aufweist.
9. Verwendung einer rekombinanten Cholesterinoxidase gemäß einem der Ansprüche 7 oder 8 in einem enzymatischen Test zur Bestimmung von Cholesterin.

Claims

1. Active cholesterol oxidase, **characterized in that** it has the amino acid sequence shown in SEQ ID NO 2.
2. DNA which codes for a peptide with cholesterol oxidase activity having the DNA sequence shown in SEQ ID NO 1 or the DNA sequence which is complementary thereto.
3. Process for the production of a recombinant cholesterol oxidase by transformation of a suitable host cell with a DNA as claimed in claim 2 which is present cloned in a suitable expression system, culturing the transformed host cells and isolating the expressed cholesterol oxidase from the cytoplasm of the transformed cells.
4. Process as claimed in claim 3, **characterized in that** the DNA used has one of the sequences shown in SEQ ID NO 6, 8, 10, 12, 14 or 16 at the 5' end.
5. DNA as claimed in claim 2, **characterized in that** it has one of the sequences shown in SEQ ID NO 6, 8, 10, 12, 14 or 16 at the 5' end.
6. DNA as claimed in claim 5, **characterized in that** it has one of the sequences shown in SEQ ID NO 18, 20, 22, 24, 26 or 28.
7. Recombinant cholesterol oxidase, **characterized in that** it is coded by a DNA as claimed in claim 2 and has one of the sequences shown in SEQ ID NO 7, 9, 11, 13, 15 or 17 at the N-terminal end.
8. Recombinant cholesterol oxidase as claimed in claim 7, **characterized in that** it has one of the sequences shown in SEQ ID NO 21, 23, 25, 27 or 29.
9. Use of a recombinant cholesterol oxidase as claimed in one of the claims 7 or 8 in an enzymatic test for the determination of cholesterol.

Revendications

1. Cholestérol oxydase active, **caractérisée en ce qu'elle** présente la séquence d'acides aminés représentée dans SEQ ID NO: 2.
2. ADN qui code pour un peptide possédant une activité de cholestérol oxydase comprenant la séquence d'ADN représentée dans SEQ ID NO: 1 ou la séquence d'ADN complémentaire à celle-ci.
3. Procédé pour la préparation d'une cholestérol oxydase recombinante par transformation d'une cellule hôte appropriée avec un ADN selon la revendication 2, qui est présent à l'état cloné dans un système d'expression approprié,

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par mise en culture des cellules hôtes transformées et par isolation de la cholestérol oxydase exprimée à partir du cytoplasme des cellules transformées.

- 5 4. Procédé selon la revendication 3, **caractérisé en ce que** l'ADN utilisé présente, à l'extrémité 5', une des séquences représentées dans SEQ ID NO: 6, 8, 10, 12, 14 ou 16.
5. ADN selon la revendication 2, **caractérisé en ce qu'il** présente, à son extrémité 5', une des séquences représentées dans SEQ ID NO: 6, 8, 10, 12, 14 ou 16.
- 10 6. ADN selon la revendication 5, **caractérisé en ce qu'il** présente une des séquences représentées dans SEQ ID NO: 18, 20, 22, 24, 26 ou 28.
- 15 7. Cholestérol oxydase recombinante, **caractérisée en ce qu'elle** est encodée par un ADN selon la revendication 2 et **en ce qu'elle** présente, à son extrémité amino terminale, une des séquences représentées dans SEQ ID NO: 7, 9, 11, 13, 15 ou 17.
8. Cholestérol oxydase recombinante selon la revendication 7, **caractérisé en ce qu'elle** présente une des séquences représentées dans SEQ ID NO: 21, 23, 25, 27 et 29.
- 20 9. Utilisation d'une cholestérol oxydase recombinante selon l'une quelconque des revendications 7 ou 8, dans un test enzymatique pour la détermination de cholestérol.

25

30

35

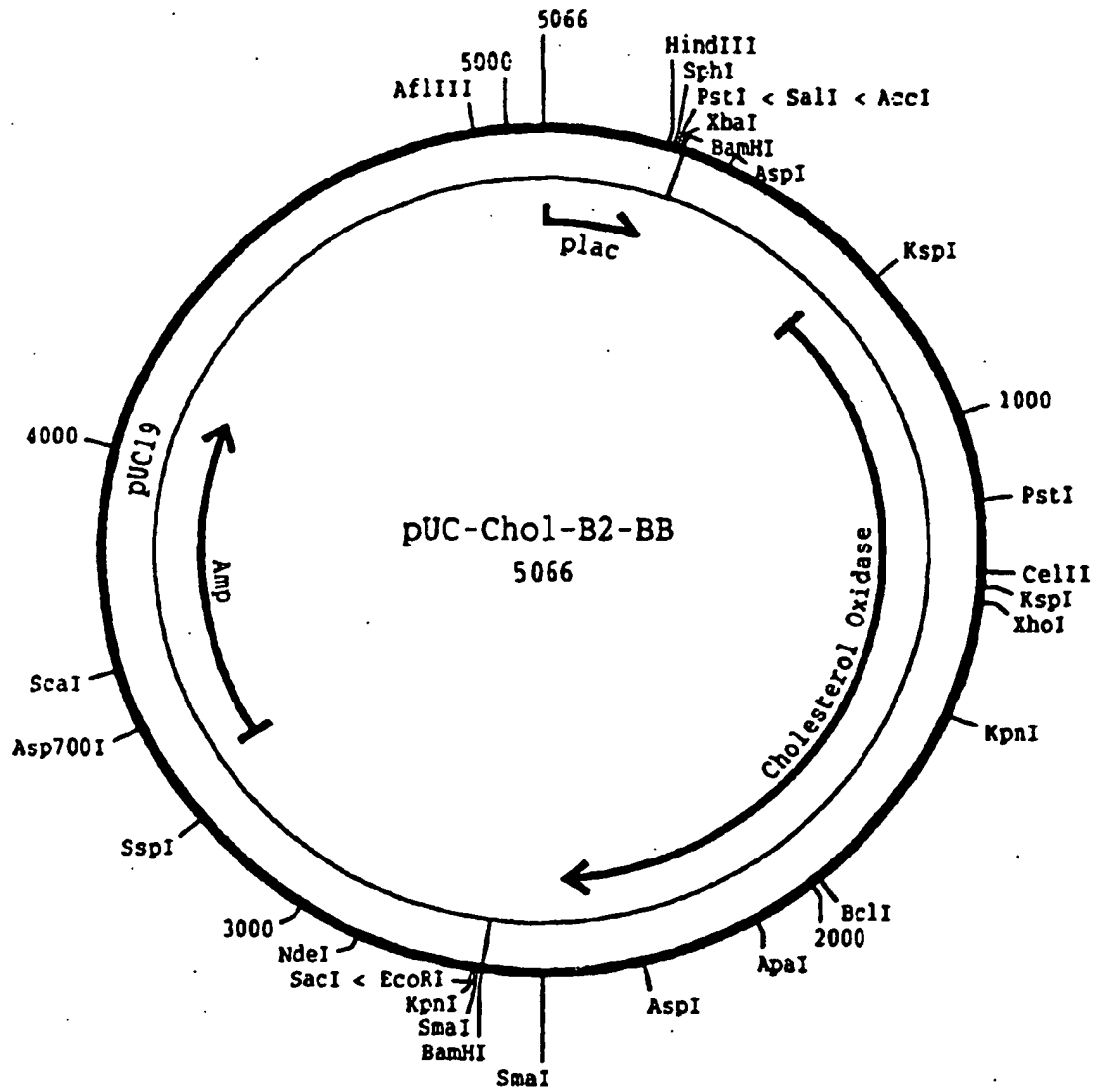
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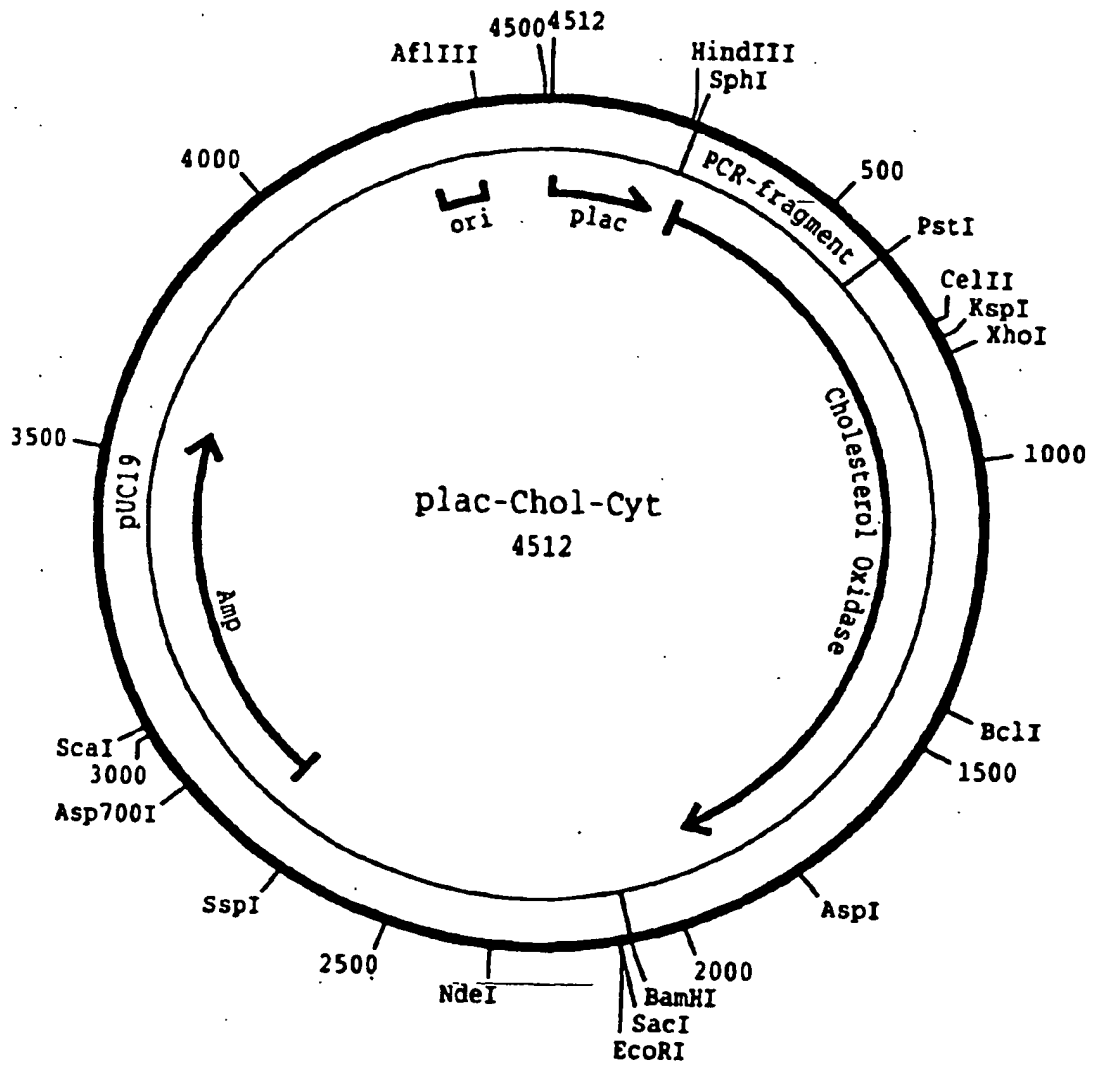
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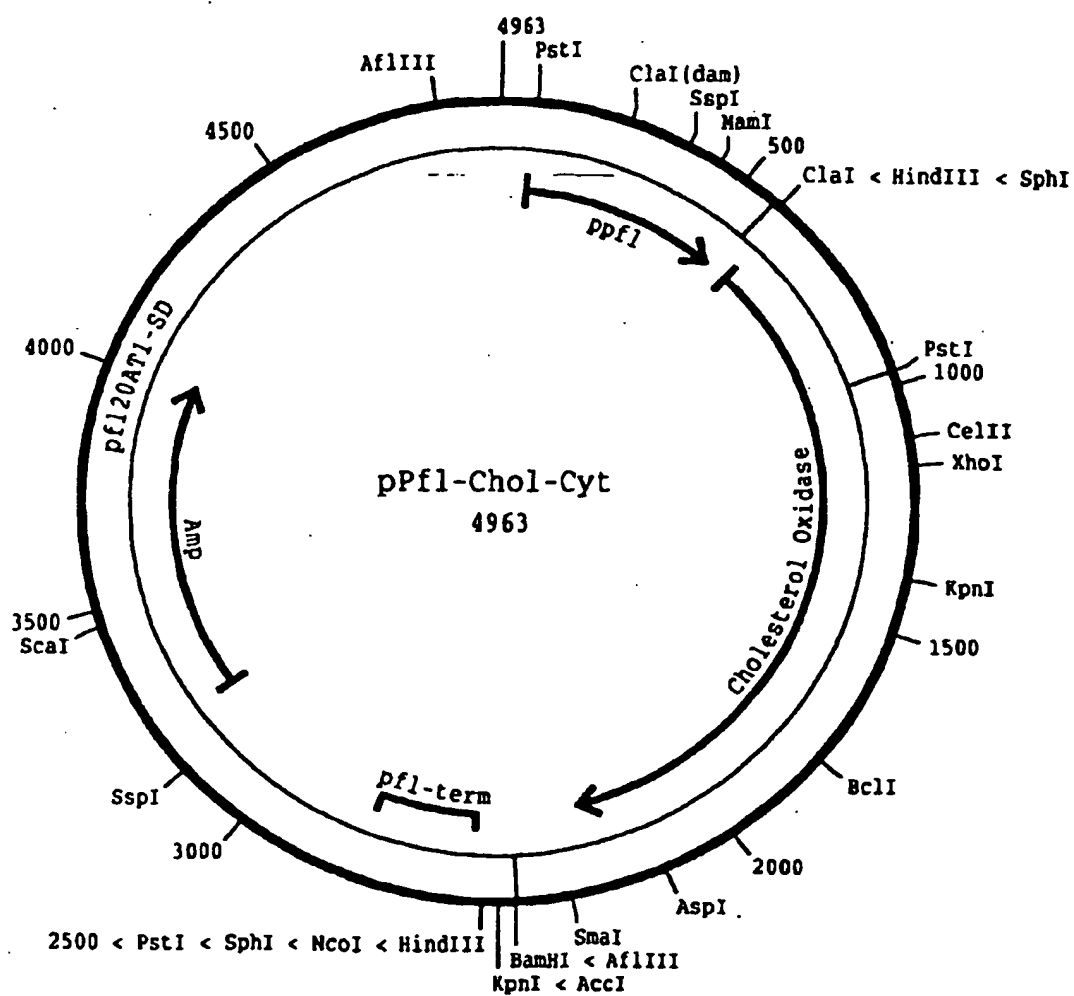
Figur 1



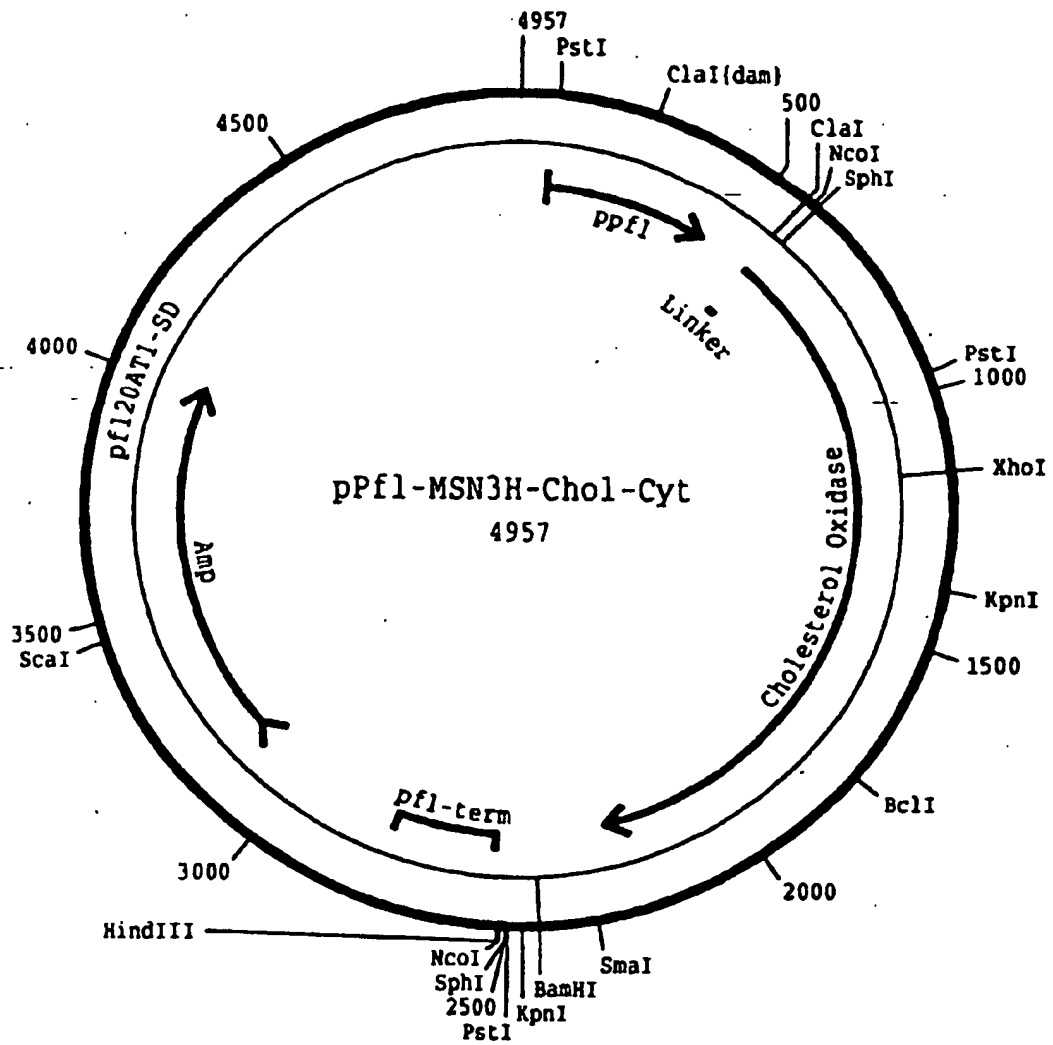
Figur 2

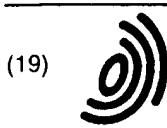


Figur 3



Figur 4





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(54) **USE OF DEOXYGALACTONOJIRIMYCIN DERIVATIVES FOR INHIBITING GLYCOLIPID SYNTHESIS**

VERWENDUNG VON DEOXYGALACTONOJIRIMYCIN DERIVATEN ZU HEMMUNG DER GLYCOLIPID SYNTHESE

UTILISATION DE DERIVES DE DESOXYGALACTONOJIRIMYCINE POUR INHIBER LA SYNTHESE DE GLYCOLIPIDES

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EP-A- 0 536 402

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DescriptionBackground of the Invention

5 This invention relates to N-alkyl derivatives of deoxygalactonojirimycin (DGJ) in which said alkyl groups contain from 3-6 carbon atoms and which are useful for selectively inhibiting glycolipid synthesis.

N-alkyl derivatives of deoxynojirimycin (DNJ) known to be inhibitors of the N-linked oligosaccharide processing enzymes, α -glucosidase I and II. Saunier et al., *J. Biol. Chem.* **257**, 14155-14166 (1982); Elbein, *Ann. Rev. Biochem.* **56**, 497-534 (1987). As glucose analogues, they also have potential to inhibit glucosyltransferases. Newbrun et al., *Arch. Oral Biol.* **28**, 516-536 (1983); Wang et al., *Tetrahedron Lett.* **34**, 403-406 (1993). Their inhibitory activity against the glucosidases has led to the development of these compounds as antihyperglycemic agents and antiviral agents. See, e.g., PCT Int'l. Appln. WO 87/03903 and U.S. Patents: 4,065,562; 4,182,767; 4,533,668; 4,639,436; 4,849,430; 5,011,829; and 5,030,638. N-alkyl derivatives of DGJ are known to be inhibitors of β -galactosidase (EP-A-0 536 402).

Brief Description of the Invention

15 In accordance with the present invention, N-alkyl derivatives of deoxygalactonojirimycin (DGJ) are used in which said alkyl contains from 3-6 carbon atoms and preferably from 4-6 carbon atoms for selectively inhibiting glycolipid synthesis. The length of the N-alkyl chain has been found to be important to said inhibitory activity since the non-alkylated DGJ and the N-methyl and N-ethyl derivatives of DGJ were each found to be inactive for such inhibition. The N-propyl derivative of DGJ was partially active. Thus, a minimum alkyl chain length of 3 carbon atoms has been found to be essential for efficacy. Illustratively, the biosynthesis of glycolipids in cells capable of producing glycolipids can be selectively inhibited by treating said cells with a glycolipid inhibitory effective amount of any of these novel compounds.

25 The active N-alkyl derivatives of DGJ have a significant advantage since, unlike the previously described N-alkyl derivatives of DNJ, they selectively inhibit biosynthesis of glycolipids without effect either on the maturation of N-linked oligosaccharides or lysosomal glucocerebrosidase. For example, in contrast to N-butyl DNJ, the N-butyl DGJ of the present invention surprisingly does not inhibit the processing α -glucosidases I and II or lysosomal β -glucocerebrosidase. Likewise, the only prior reported experimental evidence using deoxygalactonojirimycin indicates that N-alkylation (N-heptyldeoxygalactonojirimycin) provides a modest increase in the affinity towards certain β -glucosidases [Legler & Pohl, *Carb. Res.* **155**, 119 (1986)]. The inhibitory results described herein for the N-alkylated deoxygalactonojirimycin analogues in which the alkyl contains from 3 to 6 carbon atoms were unexpected in view of the corresponding activity of related iminosugar compounds.

Further uniqueness of the present invention is seen by the finding that the exemplary N-butyl and N-hexyl derivatives of DGJ completely prevented glycolipid biosynthesis, whereas the N-butyl derivatives of mannose, fucose and N-acetylglucosamine were without effect on glycolipid biosynthesis.

35 The inhibitory effect of these compounds on the biosynthesis of glycolipids is illustrated herein in the myeloid cell line HL-60 and in the lymphoid cell line H9. These are well-known, widely distributed and readily available human cell lines. For example, HL-60 cells are promyelocytic cells described by Collins et al., *Nature* **270**, 347-349 (1977). They are also readily available from the American Type Culture Collection, Rockville, MD, U.S.A., under accession number ATCC CCL 240. H9 cells are of lymphoid origin described by Gallo and Popovic, *Science* **224**, 497-500 (1984). They are also readily available from the same depository under accession number ATCC HTB 176.

45 The inhibition of glycolipid biosynthesis by these N-alkyl derivatives of DGJ is further demonstrated herein by the reduction of the binding of cholera toxin to the illustrative cell line H9 when cultured in the presence of N-butyl DGJ. These compounds thus are also useful as anti-microbial agents by inhibiting the surface expression on glycolipid receptors for bacteria and bacterial toxins as illustrated hereinafter in Tables 1 and 2, respectively.

55 The inhibitory effect upon the biosynthesis of glycolipids is further illustrated by the ability of the N-butyl and N-hexyl derivatives of DGJ to offset glucoceramide accumulation in a standard, state-of-the-art in vitro model of Gaucher's disease. In this model, the murine macrophage cell line WEHI-3B was cultured in the presence of an irreversible glucocerebrosidase inhibitor, conduritol β epoxide (CBE), to mimic the inherited disorder found in Gaucher's disease. WEHI-3B cells are described in *Cancer Res.* **37**, 546-550 (1977), and are readily available from the American Type Culture Collection, Rockville, MD, under accession number ATCC TIB 68. The compounds of the invention prevent lysosomal glycolipid storage which is useful for the management of Gaucher's disease and other glycolipid storage disorders as illustrated hereinafter in Table 3. Gaucher's disease is an autosomal recessive disorder characterized by an impaired ability to degrade glucocerebroside (glucosyl ceramide, Glc-Cer) due to mutations in the gene encoding β -glucocerebrosidase (β -D-glucosyl-N-acylsphingosine glucohydrolase, EC 3.2.1.45). This defect results in the lysosomal accumulation of Glc-Cer in cells of the macrophage-monocyte system [Barranger and Ginns, in *The Metabolic Basis of Inherited Diseases*, ed. Scriver et al., pp. 1677-1698, McGraw-Hill, New York, (1989); Beutler, *Science* **256**, 794-799 (1992)]. By slowing the rate of glycolipid synthesis, the impaired catabolism of Glc-Cer can be offset, thereby

leading to the maintenance of a balanced level of Glc-Cer.

The clinical management of Gaucher's disease currently relies upon either symptomatic treatment of patients or enzyme replacement therapy [Beutler, *Proc. Natl. Acad. Sci. USA* 90, 5384-5390 (1993)]. In view of the prohibitive cost of enzyme replacement therapy and the requirement for intravenous administration of glucocerebrosidase, an orally available alternative therapy based around substrate deprivation constitutes a useful alternative. Since NB-DGJ exhibits fewer complicating enzyme inhibitory characteristics than α - and β -glucosidase inhibitors, it constitutes a preferable alternative to NB-DNJ for therapeutic management of Gaucher's disease and other glycolipid storage disorders.

Detailed Description of the Invention

While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the invention, it is believed that the invention will be better understood from the following illustrative detailed description of the invention taken in conjunction with the accompanying drawings in which:

FIG. 1 shows by one dimensional thin layer chromatography (1D-TLC) a comparison of N-alkylated imino sugars as inhibitors of glycolipid biosynthesis. 1D-TLC separation was made of HL-60 total cellular lipids labelled with [14 C]-palmitic acid. Cells were treated with either 0.5 mM N-butyl deoxynojirimycin (NB-DNJ), N-butyl deoxymannojirimycin (NB-DMJ), N-butyl deoxygalactonojirimycin (NB-DGJ) or N-butyl 2-acetamido-1,2,5-trideoxy-1,5-imino-D-glucitol (NB-NAG) or untreated (UT). Glycolipid biosynthesis inhibition was detected by the lack of Glc-Cer, gangliosides and an unknown species (indicated with arrows). Glc-Cer migration was confirmed by inclusion of a [14 C]-Glc-Cer standard on the TLC. The radiolabelled lipid species were visualized by autoradiography.

FIG. 2, in three parts, A, B and C, shows 2D-TLC analysis of HL-60 cells treated with either NB-DNJ or NB-DGJ. 2D-TLC separation was made of total HL-60 lipids labelled with [14 C]-palmitic acid. Cells were treated with either 0.5 mM NB-DNJ or NB-DGJ or untreated (UT). Lipids were assigned as follows (untreated cells, lefthand panel, FIG. 2A): 1, gangliosides; 2, lysophosphatidylcholine; 3, ceramide phosphorylcholine; 4, ceramide phosphorylethanolamine; 5, phosphatidylcholine; 6, phosphatidylinositol; 7, phosphatidylethanolamine; 8, phosphatidylglycerol; 9, diglycosylceramide; 10, monoglycosylceramide; 11, cholesterol/fatty acids/neutral lipids; N and N* are unknowns; and 0 is the sample origin. Following NB-DNJ and NB-DGJ treatment (middle and righthand panels, FIGS. 2B and 2C, respectively) species 1 (gangliosides); 9 (diglycosylceramide); 10 (monoglycosylceramide) and N* (unknown) were absent. The radiolabelled lipids were visualized by autoradiography.

FIG. 3 shows the dose dependent effects of NB-DNJ and NB-DGJ on glycolipid biosynthesis. 1D-TLC analysis was made of total cellular lipids. HL-60 cells were labelled with [14 C]-palmitic acid in the presence or absence (UT) of NB-DNJ or NB-DGJ at the indicated concentrations (μ M). The migration position of [14 C]-Glc-Cer is indicated by arrows. The lipids were visualized by autoradiography.

FIG. 4, in two parts, A and B, shows the effects of increasing DNJ and DGJ N-alkyl chain length on inhibition of glycolipid biosynthesis. 1D-TLC analysis was made of total cellular lipids. HL-60 cells were treated with [14 C]-palmitic acid in the presence or absence (UT) of either DNJ, or the N-ethyl, N-methyl, N-propyl, N-butyl and N-hexyl derivatives of DNJ (lefthand panel, FIG. 4A) or DGJ, or the N-ethyl, N-methyl, N-propyl, N-butyl and N-hexyl derivatives of DGJ (righthand panel, FIG. 4B) at 0.5 mM concentration. The migration position of [14 C]-Glc-Cer is indicated with arrows. The lipids were visualized by autoradiography.

FIGS. 5 and 6 show the analysis of NB-DNJ and NB-DGJ in an in vitro Gaucher's disease model. Specifically, FIG. 5 shows the 1D-TLC analysis of glycolipids from WEHI-3B cells treated with either NB-DNJ or NB-DGJ, at the indicated concentrations (μ M), and visualized by chemical detection (see methods hereinafter).

FIG. 6, in eight parts, A through H, shows the transmission electron microscopy of WEHI-3B cell lysosomes: FIG. 6A, untreated; FIG. 6B, conduritol β epoxide (CBE) treated; FIG. 6C, CBE and 500 μ M NB-DNJ; FIG. 6E, CBE and 50 μ M NB-DNJ; FIG. 6G, CBE and 5 μ M NB-DNJ; FIG. 6D, CBE and 500 μ M NB-DGJ; FIG. 6F, CBE and 50 μ M NB-DGJ; FIG. 6H, CBE and 5 μ M NB-DGJ. The scale bar at the lower right hand corner of FIG. 6H is applicable to all of FIGS. 6A through H and represents 0.1 μ M.

FIG. 7 shows the effect of NB-DGJ on N-linked oligosaccharide processing. Specifically, it shows Endo H sensitivity of gp120 expressed in Chinese hamster ovary (CHO) cells in the presence or absence (-) of either NB-DNJ or NB-DGJ (0.5 mM and 5 mM). The arrows indicate the molecular weight of the untreated gp120 (120 kDa) and post endo H digestion (60 kDa). An additional band of low molecular weight (approximately 60 kDa) was present in some lanes and is a non-specific protein precipitated by the solid phase matrix.

FIG. 8 is a graphical representation that shows, in three parts, A, B and C, the effect of imino sugar analogues on glycolipid and glycoprotein metabolizing enzyme activity. Enzyme activity was determined in the presence of the following test compounds: DNJ, (\diamond); NB-DNJ, (\blacksquare); DGJ, (\blacktriangle); NB-DGJ, (\bullet) at concentrations shown (see methods hereinafter). FIG. 8A, UDP-glucose:N-acylsphingosine glucosyltransferase; FIG. 8B, β -glucocerebrosidase; FIG. 8C, processing α -glucosidase. Enzymatic activity is expressed as a percentage of control reactions that contained no test compound.

FIG. 9 shows the laser desorption mass spectrometry of N-butyl deoxygalactonojirimycin with a molecular weight of 220 (M+H) and obtained in greater than 95% purity.

FIG. 10 shows the ^1H NMR spectrum of N-butyl deoxygalactonojirimycin.

FIG. 11 is a graphical representation of a cholera toxin binding assay and shows on the y-axis the % reduction in cholera toxin binding sites per cell for H9 cells in which the cholera toxin was fluorescein conjugated and in which the levels of binding to the cell surfaces of untreated (ut) cells and cells treated with N-butyl deoxygalactonojirimycin (NB-DGJ) or, for comparison, N-butyl deoxynojirimycin (NB-DNJ), at various levels shown on the x-axis (mg/ml), were measured by flow cytometry.

In order to further illustrate the invention, the following detailed examples were carried out although it will be understood that the invention is not limited to these specific examples or the details therein.

EXAMPLES

MATERIALS & METHODS

Compounds:

N-Butyldeoxynojirimycin (NB-DNJ) was obtained from Searle/Monsanto (St. Louis, MO, U.S.A.).

Deoxygalactonojirimycin (DGJ), deoxyfuconoijirimycin (DFJ), deoxymannoijirimycin (DMJ), and 2-acetamido-1,2,5-trideoxy-1,5-imino-D-glucitol (NAG), were obtained from Cambridge Research Biochemicals (Northwich, Cheshire, U.K.). DGJ, DFJ, DMJ and NAG were reductively N-alkylated in the presence of palladium black under hydrogen using the appropriate aldehyde by conventional procedure as described by Fleet et al., *FEBS Lett.* 237, 128-132 (1988). The reaction mixture was filtered through Celite and the solvent removed by evaporation under vacuum. The resulting N-alkylated analogues were purified by ion-exchange chromatography (Dowex® AG50-X12, H⁺ form) in 2M NH₃ (aq) and the solvent removed by evaporation. These compounds were lyophilised and analysed by 1D ^1H NMR at 500 MHz on a Varian Unity 500 spectrophotometer and by matrix assisted laser desorption (Finnegan). All compounds synthesised were greater than 95% pure. The following are representative examples of the synthesis of the foregoing N-alkylated compounds as used hereinafter.

Example 1

In a representative example of the preparation of the N-butyl deoxygalactonojirimycin, 30 mg (184 μmol) of deoxygalactonojirimycin was dissolved in 1 ml of 50 mM sodium acetate buffer, pH 5.0, to which 20 mg of palladium black was added. A hydrogen atmosphere was maintained in the reaction vessel and 100 μl (1.1 mmol) of butyraldehyde was introduced. The reaction was stirred for 16 hr. at room temperature (ca. 20°C). The reaction was stopped by filtration through a bed (1 g) of Celite (30-80 mesh) and the reaction products were separated by chromatography using a column containing 4 ml of packed Dowex® AG50-X12 (H⁺ form) resin. The N-butyl deoxygalactonojirimycin was eluted from the chromatography column with 2M ammonia. Its molecular mass was 220 (M+H) as determined by laser desorption mass spectrometry and its chemical structure was confirmed by 1D ^1H NMR as shown in Figures 9 and 10, respectively.

Example 2

The synthesis procedure and compound analysis of Example 1 was repeated except that caproaldehyde was substituted for an equivalent amount of butyraldehyde for analogous preparation of N-hexyl deoxygalactonojirimycin. Its molecular mass was 248 (M+H) as determined by laser desorption mass spectrometry and its chemical structure was confirmed by 1D ^1H NMR.

Example 3

The synthesis procedure and compound analysis of Example 1 was repeated except that propanoyl aldehyde was substituted for an equivalent amount of butyraldehyde for analogous preparation of N-propyl deoxygalactonojirimycin. Its molecular mass was 206 (M+H) as determined by laser desorption mass spectrometry and its chemical structure was confirmed by 1D ^1H NMR.

The N-alkylated deoxygalactonojirimycin compounds prepared in the foregoing illustrative Examples 1 to 3 were obtained in overall yields of 68-74% based on the starting deoxygalactonojirimycin and were greater than 95% pure.

Enzymes and Enzyme Assays:

Porcine liver α -glucosidase I and rat liver α -glucosidase II were purified to homogeneity and assayed by conventional procedure using a [14 C]-glucose labelled Glc₃Man₉GlcNAc₂ substrate as previously described by Karlsson et al., *J. Biol. Chem.* **268**, 570-576 (1993).

β -D-Glucosyl-N-acylsphingosine glucosylhydrolase (glucocerebrosidase) was isolated from human placenta and purified to homogeneity according to published standard methods [Furbish et al., *Proc. Natl. Acad. Sci. USA* **74**, 3560-3563 (1977); Dale and Beutler, *ibid.* **73**, 4672-4674 (1976)]. Glucocerebrosidase activity was measured by adding enzyme (5-50 μ l) to a sonicated suspension of buffer (50 μ l of 50 mM sodium citrate/sodium phosphate buffer, pH 5.0) containing glucosyl ceramide (1 mM), Triton® X-100 non-ionic surfactant (0.25% v/v) and sodium taurodeoxycholate (0.6% v/v) that had been previously dried under nitrogen from chloroform:methanol (2:1 v/v) solutions. After incubation at 37°C for 15-60 min., the reaction was stopped by the addition of 500 μ l of chloroform:methanol and the phases separated by centrifugation. The upper phase was washed twice with Folch theoretical lower phase [Folch et al., *J. Biol. Chem.* **226**, 497-509 (1957)] desalted using AG50-X12 ion-exchange resin and dried under vacuum. The reaction products were separated by high performance anion exchange chromatography (Dionex BioLC System) and detected by pulsed amperometry. The amount of enzyme-released glucose was calculated from peak areas by applying experimentally determined response factors for glucose relative to an included reference monosaccharide [Butters et al., *Biochem. J.* **279**, 189-195 (1991)].

UDP-glucose:N-acylsphingosine glucosyltransferase (EC 2.4.1.80) activity was measured in rat brain homogenates and mouse macrophage tissue cultured cell (WEHI-3B) homogenates using a method adapted as follows from published conventional procedures [Vunnam and Radin, *Chem. & Phys. of Lipids* **26**, 265-278 (1980); Shukla and Radin, *Arch. Biochem. Biophys.* **283**, 372-378 (1990)]: Dioleoylphosphatidylcholine and cerebroside liposomes containing 200 nmol ceramides Type IV (Sigma) were added to a reaction mixture (100 μ l) composed of 40 mM 2-[N-morpholino]ethanesulfonic acid (MES) buffer, pH 6.5, 5 mM MnCl₂, 2.5 mM MgCl₂, 1 mM NADH and 8 μ M UDP-[14 C]-glucose (318 mCi/mmol, Amersham International, Amersham, U.K.). After incubation at 37°C for 1-2 hr. the reaction was stopped by the addition of EDTA (25 mM) and KCl (50 mM). Radiolabelled glycolipids were extracted with 500 μ l of chloroform:methanol (2:1 v/v) for 10 min. and the phases separated. The lower phase was washed twice with Folch theoretical upper phase and portions taken for scintillation counting. When imino sugars were tested for inhibitory activity, these were added at appropriate concentrations to homogenates and preincubated for 10 min. before sonication with ceramide containing liposomes. Control reactions were performed with liposomes containing no ceramide to measure the activity of transfer to endogenous acceptors.

Glycolipid Analysis:

HL-60 cells were cultured by conventional procedure as previously described by Platt et al., *Eur. J. Biochem.* **208**, 187-193 (1992). HL-60 cells at 5 x 10⁴ cells/ml were cultured in the presence or absence of imino sugars for 24 hr. For labelling, the two dimensional thin layer chromatography (2D-TLC) conventional method of Butters and Hughes was followed [In Vitro **17**, 831-838 (1981)]. Briefly, [14 C]-palmitic acid (56.8 mCi/mmol, ICN/Flow) was added as a sonicated preparation in foetal calf serum (FCS, Techgen, London, U.K., 0.5 μ Ci/ml) and the cells cultured for a further 3 days maintaining the imino sugars in the medium. The cells were harvested, washed three times with phosphate buffered saline (PBS), extracted in 1 ml chloroform:methanol (2:1 v/v) and separated by 1 dimensional TLC, loading equal counts (1D-TLC, chloroform:methanol:water (65:25:4)). For two dimensional separations the one dimensional separation was performed as described above, the plate dried overnight under vacuum and separated in the second dimension using a solvent of tetrahydrofuran: dimethoxymethane:methanol:water (10:6:4:1). Plates were air dried and exposed to Hyperfilm-MP high performance autoradiography film (Amersham).

Cell Culture and Metabolic Labelling:

The culture of CHO cells expressing soluble recombinant gp120 (from Dr. P. Stevens, MRC AIDS Directed Programme Reagent Project) and the radiolabelling of these cells was carried out by conventional procedure as described by Karlsson et al., *J. Biol. Chem.* **268**, 570-576 (1993). Briefly, CHO cells were harvested mechanically, washed three times with phosphate buffered saline, 0.1M pH 7.2 (PBS) and resuspended in methionine- and cysteine-free RPMI-1640 medium (ICN-Flow Laboratories, High Wycombe, Bucks, U.K.) supplemented with 1% dialysed FCS. Cells (10⁷/ml) were preincubated in the presence or absence of NB-DNJ or NB-DGJ for 1 hr prior to the addition of 100 μ Ci/ml Tran³⁵S-label (ICN-Flow) for 4 hr. The supernatants were collected and concentrated tenfold using a 30 kDa cut-off membrane (Amicon, Danvers, MA, U.S.A.).

Immunoprecipitation:

Immunoprecipitations were performed by conventional procedure as described by Karlsson *supra*. Supernatants were incubated with the mAb ABT 1001 monoclonal antibody (American Biotechnologies Inc., Cambridge, MA, U.S. A.) at 0.5 µg/100 µl of supernatant for 30 min. at room temperature followed by sheep anti-mouse IgG1-coated magnetic beads (DynaL Ltd., Wirral, Merseyside, U.K., 1.2×10^7 beads per sample) for 1 hr. at 4°C. The beads were washed three times with 2% Triton® X-100 in PBS and three times with PBS. Gp120 was eluted in 100 µl reducing SDS-PAGE sample buffer with heating (95°C, 5 min.). Each sample was divided into two equal aliquots and 25 µl of dH₂O added to give a final volume of 50 µl. To one half of each sample 2 µl of endoglycosidase H (endo H, 1 unit/ml, Boehringer Mannheim Ltd., Lewes, Sussex, U.K.) was added and the other half left untreated. Digestion was performed at 37°C for 18 hr. and terminated by the addition of 50 µl of SDS-PAGE reducing sample buffer (95°C, 5 min.).

Glycopeptide Analysis:

HL-60 and BW5147 cells were cultured in RPMI-1640 and 10% FCS. The cells were incubated for 30 min. in the presence or absence of 2 mM NB-DNJ or NB-DGJ in reduced glucose RPMI-1640 medium (Flow), supplemented with 1% dialysed FCS. [³H]-mannose (16.5 Ci/mmol, Amersham) was added at 200 µCi/ml and the cells cultured for a further 3 hr. Washed cell pellets were resuspended in 50 mM TrisHCl buffer, pH 7.5, containing 10 mM CaCl₂ and 0.02% sodium azide and heated at 100°C for 5 min. After cooling, Pronase® enzyme was added to 0.04% (w/v final concentration) and incubated for 96 hr. at 37°C under toluene with aliquots of Pronase® added at each 24 hr. period. The digestion was stopped by boiling for 5 min., and glycopeptides recovered by centrifugation at 13000 g for 10 min. Samples were fractionated by Con A-Sepharose® chromatography according to conventional procedure of Foddy et al., *Biochem. J.* 233, 697-706 (1986).

In Vitro Gaucher's Disease Model:

The in vitro Gaucher's disease model was prepared as follows: WEHI-3B cells (American Type Culture Collection, Rockville, MD, U.S.A.) were maintained in logarithmic phase growth for 14 days in RPMI-1640 medium, 10% FCS, in the presence or absence of 50 µM conduritol β epoxide (CBE, Toronto Research Chemicals, Downsview, Canada) with or without NB-DNJ or NB-DGJ. Cells were passaged every 3 days and compound concentrations maintained throughout. Equal cell numbers (5×10^6) were harvested, extracted in 1 ml chloroform:methanol (2:1 v/v) overnight at 4°C, the extracts centrifuged, the chloroform:methanol extract retained and the pellet re-extracted as above for 2 hr. at room temperature. Pooled extracts were dried under nitrogen, re-dissolved in 10 µl chloroform:methanol (2:1 v/v) and separated by 1D-TLC in chloroform:methanol:water (10:6:4:1). Plates were air dried and visualized using α-naphthol (1% w/v in methanol) followed by 50% (v/v) sulphuric acid.

Transmission Electron Microscopy:

Cells for electron microscopy were harvested (1×10^7 cells per treatment), washed three times in serum free RPMI-1640 medium and fixed in medium containing 2% glutaraldehyde (v/v) and 20 mM Hepes (v/v) on ice for 2 hr. Cells were washed in 0.1 M cacodylate buffer containing 20 mM calcium chloride (w/v). Cells were post-fixed with 1% osmium tetroxide in 25 mM cacodylate buffer (w/v) containing 1.5% potassium ferrocyanide (w/v) for 2 hr. on ice. Samples were dehydrated through an ethanol series, transferred to propylene oxide and embedded in Embed 800 (Electron Microscopy Sciences, PA, U.S.A.). The sections were stained with uranyl acetate/lead citrate and observed with a Hitachi 600 microscope at 75 kv.

Analysis of cholera toxin binding to the H9 human lymphoid cell line following three days treatment with NB-DNJ or NB-DGJ:

Methods: Cells were maintained in logarithmic phase growth in RPMI-1640 medium. Cholera toxin B chain (Sigma) was conjugated to fluorescein isothiocyanate (Sigma) and flow cytometric analysis was carried out by conventional procedure as described by Platt et al., *Eur. J. Biochem.* 208, 187-193 (1992). Analysis was performed on a FACScan Cytometer (Becton Dickinson, Sunnyvale, CA, USA). Data on viable cells were collected on a four decade log₁₀ scale of increasing fluorescence intensity. The data are presented as percent reduction in cholera toxin bindings sites per cell on the y-axis against compound concentration on the x-axis. The specificity of cholera toxin : cell surface binding was established by inhibiting this interaction with a one hundred fold molar excess of GM1 derived oligosaccharide, GalβGalNAcβ4(NeuAcα3)Galβ4Glcβ3Cer.

RESULTS

Comparison of N-alkylated imino sugars as inhibitors of glycolipid biosynthesis:

The glucose analogue, NB-DNJ and four pyranose analogues (NB-DMJ, mannose analogue; NB-DFJ, fucose analogue; NB-DGJ, galactose analogue; and NB-NAG, N-acetylglucosamine analogue) were assessed by the above methods for their capacities to inhibit the metabolic incorporation of radiolabelled palmitate into glycolipids in HL-60 cells at a 500 μ M compound concentration using 1D-TLC analysis (Fig. 1). In addition to NB-DNJ, the only analogue which specifically inhibited glycolipid biosynthesis was NB-DGJ. All other analogues were without effect. Both NB-DNJ and NB-DGJ inhibited the biosynthesis of Glc-Cer, gangliosides and an unknown lipid species in agreement with the previous observations with NB-DNJ described in copending application Ser. No. 08/061,645. To confirm that NB-DNJ and NB-DGJ had comparable effects on the complete spectrum of glycolipids in this cell line, 2D-TLC was performed to resolve further the individual glycolipid species (Fig. 2). A total depletion of glycolipid species was achieved with both 500 μ M NB-DNJ and NB-DGJ. Specifically, gangliosides, the unknown lipid (N*) and both the mono and dihexaside species were absent following treatment with either compound. Phospholipid composition and relative abundance were comparable, irrespective of treatment, consistent with the previous observations in copending application Ser. No. 08/061,645 that N-alkylated imino sugars have no effect on sphingomyelin or phospholipid biosynthesis. When the two analogues were compared at a range of concentrations by 1D-TLC (Fig. 3) both analogues exhibited complete glycolipid inhibition between 50 μ M and 500 μ M concentrations, although partial inhibition occurred with both compounds at concentrations as low as 0.5-5 μ M. Both analogues were non-cytotoxic in the dose range tested.

Effects of increasing DNJ and DGJ N-alkyl chain length on inhibition of glycolipid biosynthesis:

A series of N-alkylated DNJ and DGJ derivatives were compared for their abilities to inhibit glycolipid biosynthesis (Fig. 4A and 4B, respectively) by 1D-TLC. The non-alkylated imino sugars and the N-methyl DNJ, N-ethyl DNJ, N-methyl DGJ and N-ethyl DGJ had no effect on glycolipid biosynthesis. The N-propyl analogues of both parent compounds showed partial inhibitory activity, whereas the N-butyl and N-hexyl derivatives of DNJ and DGJ completely inhibited glycolipid biosynthesis, as determined by the loss of detectable Glc-Cer. These data were therefore in agreement with the data from the previous application Ser. No. 08/061,645 (where the N-methyl derivative was compared with N-butyl and N-hexyl DNJ). There is a minimal N-alkyl chain length requirement to achieve full inhibition of glycolipid biosynthesis, with butyl and hexyl being optimal.

Analysis of NB-DGJ in an in vitro Gaucher's disease model:

The WEHI-3B murine macrophage cell line can be induced to resemble Gaucher's cells by treatment with the irreversible glucocerebrosidase inhibitor CBE. NB-DNJ and NB-DGJ were compared in their ability to prevent the accumulation of Glc-Cer in this system (Fig. 5). Both analogues prevented CBE induced glycolipid storage in the 5-50 μ M dose range. These data therefore demonstrate that NB-DGJ is as effective as NB-DNJ in preventing glycolipid storage in this in vitro Gaucher's disease model. The status of the lysosomes from cells treated with either NB-DNJ or NB-DGJ was assessed by transmission electron microscopy (Fig. 6). It was found that both analogues prevented the glycolipid accumulation observed in the lysosomes of cells treated with CBE.

Specificity of NB-DGJ for the glycolipid biosynthetic pathway:

The CHO cell line is unique in that it lacks significant levels of the Golgi endomannosidase which acts to circumvent α -glucosidase I and II inhibition [Karlsson et al., *J. Biol. Chem.* 268, 570-576 (1993); Hiraizumi et al., *J. Biol. Chem.* 268, 9927-9935 (1993)]. As a consequence, it offers an unambiguous cellular system in which to test α -glucosidase inhibition. NB-DNJ was previously tested in this cell line expressing recombinant gp120 and it was found that it results in the maintenance of glucosylated high mannose oligosaccharides on gp120 which are fully sensitive to endo H [Karlsson et al., *supra*].

Analysis of the N-linked oligosaccharides of gp120 expressed in CHO cells was performed in the presence or absence of NB-DNJ or NB-DGJ (Fig. 7). Treatment of CHO cells with 0.5 mM or 5 mM NB-DNJ resulted in fully endo H sensitive gp120 N-linked glycans in contrast to the untreated gp120 which was partially sensitive to endo H. This partial sensitivity of untreated gp120 to endo H is because gp120 carries approximately fifty percent high mannose N-linked oligosaccharides per molecule [Mizuochi et al., *Biochem. J.* 254, 599-603 (1988); Mizuochi et al., *Biomed. Chrom.* 2, 260-270 (1988)]. However, when the galactose analogue, NB-DGJ, was tested in this system, at 0.5 mM and 5 mM concentrations, gp120 remained partially sensitive to endo H and was indistinguishable from the untreated gp120 molecules. This suggested that the galactose analogue was not acting as an inhibitor of α -glucosidases I and II.

To examine the effect on endogenous glycoprotein synthesis, radiolabelled glycopeptides were isolated from treated HL-60 and murine BW5147 cells and analysed for their affinity for Con A-Sepharose®. This procedure efficiently resolves tetra- and tri-antennary complex N-glycans from bi-antennary and high mannose/hybrid N-glycans [Foddy et al., *Biochem. J.* **233**, 697-706 (1986)]. Addition of NB-DNJ changes the affinity of glycopeptides eluting from the Con A-Sepharose® column (Table 4) as a result of processing glucosidase inhibition. Thus the proportion of unbound glycans (tetra- and tri-antennary species) decreases, and a corresponding increase is found in the proportion of high mannose/hybrid glycans that are tightly bound to Con A-Sepharose® and eluted with 500 mM methylmannoside. Similar gross changes in glycopeptide composition following treatment with α -glucosidase inhibitors are well established [Moore and Spiro, *J. Biol. Chem.* **265**, 13104-13112 (1990)]. The galactose analogue, NB-DGJ, showed an unchanged glycopeptide profile by Con A-Sepharose® chromatography (Table 4). To confirm these data, glucosidase inhibition was measured directly in vitro using a mixture of purified α -glucosidases I and II (Fig. 8). Whereas NB-DNJ inhibited glucosidase I and II with an IC_{50} of 0.36 μ M, NB-DGJ was only weakly inhibitory (IC_{50} of 2.13 mM, Table 5). These data provide substantial evidence that in both in vitro α -glucosidase assays and in intact cellular system assays NB-DGJ does not inhibit N-linked oligosaccharide processing.

DNJ and its N-alkylated derivatives are inhibitors of the purified lysosomal glucocerebrosidase enzyme required for the cleavage of Glc-Cer to glucose and ceramide [Osiecki-Newman et al., *Biochim. Biophys. Acta* **915**, 87-100 (1987)]. In recent tests with the in vitro Gaucher's disease model in co-pending application Ser. No. 08/061,645, it was observed that WEHI-3B cells incubated in the absence of CBE but in the presence of NB-DNJ accumulated Glc-Cer. It was therefore apparent that the N-butyl derivative of DNJ was also acting as an inhibitor of glucocerebrosidase in a cellular environment. The inhibitory activity of NB-DNJ and NB-DGJ was therefore directly measured to investigate quantitatively their capacities to inhibit human placental glucocerebrosidase (Table 5). NB-DNJ provided moderate inhibition of catalysis with an IC_{50} of 0.52 mM while NB-DGJ did not inhibit enzyme activity even at the highest concentration tested (1 mM). In terms of percent enzyme inhibition achieved with the two analogues, 1 mM NB-DNJ resulted in 90% inhibition while 1 mM NB-DGJ was non-inhibitory (Fig. 8), thereby further confirming the advantageous and unexpected selective inhibitory activity of NB-DGJ compared to that of NB-DNJ.

Inhibition of UDP-glucose:N-acylsphingosine glucosyltransferase:

The determination of transferase activity using rat brain or mouse macrophage tissue cultured cells followed saturation kinetics for both exogenously added ceramide acceptor and UDP-glucose donor. Under these conditions both N-butylated DNJ and DGJ were moderate inhibitors of glucose transfer, (IC_{50} 2.95 mM and 60.88 mM, respectively, Table 5) whereas their unmodified parent homologues were not inhibitory at the highest concentration tested 6.1 and 5.0 mM, respectively, Fig. 8).

Analysis of cholera toxin binding to the H9 human lymphoid cell line treated with NB-DGJ:

The activity of the representative N-butyl deoxygalactonojirimycin (NB-DGJ) for inhibiting the surface expression of glycolipid receptors for bacteria and bacterial toxins was illustrated by subjecting H9 cells to cholera toxin binding sites in the presence of varying concentrations of the NB-DGJ. As a specific probe, advantage was taken of the GM1 binding specificity of the cholera toxin B chain [van Heyningen, *Nature* **249**, 415-417 (1974); Karlsson, *Ann. Rev. Biochem.* **58**, 309-350 (1989)]. The binding of cholera toxin to H9 cells cultured in the presence of NB-DGJ was reduced by approximately 70% (Fig. 11). This was consistent with the loss of GM1 from the cell surface and provided further evidence for the inhibition of glycolipid biosynthesis by NB-DGJ, even though by comparison it was less than the approximately 90% reduction (Fig. 11) obtained with the N-butyl deoxynojirimycin (NB-DNJ). These results demonstrate that the imino sugar derivatives have use as anti-microbial agents by inhibiting the surface expression of glycolipid receptors for bacteria and bacterial toxins as shown in Tables 1 and 2, respectively.

Table 1

GLYCOSPHINGOLIPID RECEPTORS FOR BACTERIAL CELLS		
Microorganism	Target Issue	Presumed Specificity
<i>E. coli</i>	Urinary	Gal α 4Gal β
<i>E. coli</i>	Urinary	GlcNAc β
<i>Propionibacterium</i>	Skin/Intestine	Gal β 4Glc β

Table 1 (continued)

GLYCOSPHINGOLIPID RECEPTORS FOR BACTERIAL CELLS		
Microorganism	Target Issue	Presumed Specificity
Several bacteria	Diverse	Gal β 4Glc β
<i>Streptococcus pneumoniae</i>	Respiratory	GlcNAc β 3Gal
<i>E. coli</i> CFA/I	Intestine	NeuAc α 8
<i>E. coli</i>	Urinary	NeuAc α 3Gal
<i>E. coli</i>	Intestine	NeuGc α 3Gal β 4Glc β CerGalNAc β 4(NeuAc α 3)Gal β 4Glc β Cer
<i>Staphylococcus saprophyticus</i>	Urinary	Gal β 4GlcNAc
<i>Actinomyces naeslundii</i>	Mouth	Gal β , GalNAc β , Gal β 3GalNAc β , GalNAc β 3Gal β
<i>Pseudomonas</i>	Respiratory	GalNAc β 4Gal
<i>Neisseria gonorrhoeae</i>	Genital	Gal β 4Glc β NeuAc α 3Gal β 4GlcNAc

Table 2

GLYCOSPHINGOLIPID RECEPTORS FOR BACTERIAL TOXINS			
Microorganism	Toxin	Target Tissue	Presumed Receptor Sequence
<i>Vibrio cholerae</i>	Cholera toxin	Small Intestine	Gal β 3GalNAc β 4(NeuAc α 3)Gal β 4Glc β Cer
<i>E. coli</i>	Heat-labile toxin	Intestine	Gal β 3GalNAc β 4(NeuAc α 3)Gal β 4Glc β Cer
<i>Clostridium tetani</i>	Tetanus toxin	Nerve	Gal β 3GalNAc β 4(NeuAc α 8NeuAc α 3)Gal β 4Glc β Cer
<i>Clostridium botulinum</i>	Botulinum toxin A and E	Nerve Membrane	NeuAc α 8NeuAc α 3Gal β 3GalNAc β 4(NeuAc α 8NeuAc α 3)Gal β 4Glc β Cer
<i>Clostridium botulinum</i>	Botulinum toxin B, C and F	Nerve Membrane	NeuAc α 3Gal β 3GalNAc β 4(NeuAc α 8NeuAc α 3)Gal β 4Glc β Cer
<i>Clostridium botulinum</i>	Botulinum toxin B	Nerve Membrane	Gal β cer
<i>Clostridium perfringens</i>	Delta toxin	Cell lytic	GalNAc β 4(NeuAc α 3)Gal β 4Glc β Cer
<i>Clostridium difficile</i>	Toxin A	Large Intestine	Gal α 3Gal β GlcNAc β 3Gal β 4Glc β Cer
<i>Shigella dysenteriae</i>	Shiga toxin	Large Intestine	Gal α 4Gal β CerGal α 4Gal β 4Glc β CerGlcNAc β 4GlcNAc

Table 2 (continued)

GLYCOPHINGOLIPID RECEPTORS FOR BACTERIAL TOXINS			
Microorganism	Toxin	Target Tissue	Presumed Receptor Sequence
<i>E. coli</i>	Vero toxin or Shiga-like toxin	Intestine	Gal α 4Gal β 4Glc β Cer

Table 3

HERIDITARY GLYCOLIPID STORAGE DISORDERS		
Disease	Lipid Accumulation	Enzyme Defect
Gaucher's	Glucocerebroside	Glucocerebroside- β -glucosidase
Ceramide Lactoside Lipidosis	Ceramide Lactoside	Ceramidelactoside- β -galactosidase
Fabry's	Ceramide Trihexoside	Ceramidetrihexoside- α -galactosidase
Tay-Sach's	Ganglioside GM2	Hexosaminidase A
Sandhoff's	Globoside and GM2	Hexosaminidase A and B
General Gangliosidosis	Ganglioside GM1	β -Galactosidase
Fucosidosis	H-isoantigen	α -Fucosidase
Krabbe's	Galactocerebroside	Galactocerebroside- β -galactosidase
Metachromatic Leukodystrophy	Sulfatide	Sulfatidase

TABLE 4

EFFECT OF IMINO SUGAR ANALOGUES ON OLIGOSACCHARIDE BIOSYNTHESIS					
Cell Line	Treatment	Tetra- & Tri- antennary	Bi-antennary	Oligomannose & hybrid	Total ^3H -mannose recovered (cpm)
HL-60	untreated	28.3	18.7	53.0	666918
	NB-DNJ	19.5	20.0	60.5	913095
	NB-DGJ	28.1	17.7	54.2	844322
BW5147	untreated	46.1	5.6	48.3	476527
	NB-DNJ	26.8	4.9	68.3	686026
	NB-DGJ	40.4	7.2	52.4	706873

Cells were radiolabelled for 4 hours with [^3H]-mannose in the presence or absence of compounds as shown above. Washed cells were exhaustively digested with Pronase® enzyme and resultant glycopeptides fractionated by Con A-Sepharose® chromatography as described hereinbefore. The percentage of radiolabelled glycopeptides that were non-bound (complex tetra- and tri-antennary N-glycans), eluted with 10 mM methylglucoside (complex bi-antennary N-glycans), or further eluted with 500 mM methylmannoside (oligomannose and hybrid N-glycans) were calculated from estimations of radioactivity recovered from pooled eluates.

TABLE 5

CONCENTRATIONS OF IMINO SUGAR ANALOGUES REQUIRED FOR THE INHIBITION OF GLYCOLIPID AND GLYCOPROTEIN METABOLISING ENZYMES				
Enzyme	DNJ	Compound NB-DNJ	IC ₅₀ values DGJ	NB-DGJ
UDP-glucose:N: acylsphingosine glucosyltransferase	-†	2.95 mM	-†	60.88 mM
β-glucocerebrosidase	2.43 mM	0.52 mM	-*	-*
α-glucosidase I and II	nd	0.36 μM	nd	2.13 mM

* not inhibitory at 1 mM concentrations of compound.

† not inhibitory at the highest concentration tested (see Fig. 8).

nd not determined.

Enzymes were assayed according to procedure described hereinbefore using concentrations of analogues shown in Fig. 8. The data from Fig. 8 were plotted on a logarithmic scale for accurate estimations of IC₅₀ values, shown above.

In addition to their use as inhibitors of glycolipid biosynthesis in cells, the inhibitory agents described herein also can be used for administration to patients afflicted with glycolipid storage defects by conventional means, preferably in formulations with pharmaceutically acceptable diluents and carriers. These agents can be used in the free amine form or in their salt form. Pharmaceutically acceptable salt derivatives are illustrated, for example, by the HCl salt. The amount of the active agent to be administered must be an effective amount, that is, an amount which is medically beneficial but does not present toxic effects which outweigh the advantages which accompany its use. It would be expected that the adult human daily dosage would normally range from about one to about 100 milligrams of the active compound. The preferable route of administration is orally in the form of capsules, tablets, syrups, elixirs and the like, although parenteral administration also can be used. Suitable formulations of the active compound in pharmaceutically acceptable diluents and carriers in therapeutic dosage form can be prepared by reference to general texts in the field such as, for example, Remington's Pharmaceutical Sciences, Ed. Arthur Osol, 16th ed., 1980, Mack Publishing Co., Easton, PA., U.S.A.

Claims

1. Use of an N-alkyl derivative of deoxygalactonojirimycin in which said alkyl contains from 3-6 carbon atoms for preparing a medicament for inhibiting the biosynthesis of glycolipids in cells capable of producing glycolipids.
2. Use according to Claim 1 in which the alkyl group contains from 4-6 carbon atoms.
3. Use according to Claim 2 in which the alkyl group is butyl.
4. Use according to Claim 2 in which the alkyl group is hexyl.
5. Use according to Claim 1 in which the inhibitory effective amount is from 50 μM to 500 μM.
6. Use according to Claim 1 in which the glycolipid is a glucoceramide based glycosphingolipid.
7. Use according to Claim 1 in which the glycolipid is a lysosomal glycolipid.
8. Use according to Claim 1 in which the glycolipid is a glucoceramide accumulating in cells affected with Gaucher's disease.
9. Use of an N-alkyl derivative of deoxygalactonojirimycin in which said alkyl contains from 3-6 carbon atoms for preparing a medicament for inhibiting the surface expression of glycolipid receptors for bacteria and bacterial toxins.
10. Use according to Claim 9 in which the alkyl group contains from 4-6 carbon atoms.

11. Use according to Claim 10 in which the alkyl group is butyl.
12. Use according to Claim 10 in which the alkyl group is hexyl.
- 5 13. Use according to Claim 9 in which the inhibitory effective amount is from 50 μ M to 500 μ M.
14. Use according to Claim 19 in which the bacterial toxin is cholera toxin.

10 Patentansprüche

1. Verwendung eines N-Alkylderivates von Deoxygalactonojirimycin, worin dieses Alkyl von 3 bis 6 Kohlenstoffatome enthält, zur Herstellung eines Medikamentes zur Inhibierung der Biosynthese von Glycolipiden in Zellen, die Glycolipide zu produzieren vermögen.
- 15 2. Verwendung nach Anspruch 1, worin die Alkylgruppe von 4 bis 6 Kohlenstoffatome enthält.
3. Verwendung nach Anspruch 2, worin die Alkylgruppe Butyl ist.
- 20 4. Verwendung nach Anspruch 2, worin die Alkylgruppe Hexyl ist.
5. Verwendung nach Anspruch 1, worin die wirksame Inhibierungsmenge von 50 μ M bis 500 μ M beträgt.
6. Verwendung nach Anspruch 1, worin das Glycolipid ein Glycosphingolipid auf der Basis von Glucoceramid ist.
- 25 7. Verwendung nach Anspruch 1, worin das Glycolipid ein lysosomales Glycolipid ist.
8. Verwendung nach Anspruch 1, worin das Glycolipid ein Glucoceramid ist, das in Zellen akkumuliert, die von Gaucher's Krankheit befallen sind.
- 30 9. Verwendung eines N-Alkylderivates von Deoxygalactonojirimycin, worin dieses Alkyl von 3 bis 6 Kohlenstoffatome enthält, zur Herstellung eines Medikamentes zur Inhibierung der Oberflächenexpression von Glycolipidrezeptoren für Bakterien und bakterielle Toxine.
- 35 10. Verwendung nach Anspruch 9, worin die Alkylgruppe von 4 bis 6 Kohlenstoffatome enthält.
11. Verwendung nach Anspruch 10, worin die Alkylgruppe Butyl ist.
12. Verwendung nach Anspruch 10, worin die Alkylgruppe Hexyl ist.
- 40 13. Verwendung nach Anspruch 9, worin die wirksame inhibierende Menge von 50 μ M bis 500 μ M beträgt.
14. Verwendung nach Anspruch 19, worin das bakterielle Toxin Cholera toxin ist.

45 Revendications

1. Utilisation d'un dérivé N-alkyle de désoxygalactonojirimycine dont ledit alkyle contient de 3 à 6 atomes de carbone pour préparer un médicament destiné à inhiber la biosynthèse de glycolipides dans des cellules capables de produire des glycolipides.
- 50 2. Utilisation selon la revendication 1, dans laquelle le groupe alkyle contient de 4 à 6 atomes de carbone.
3. Utilisation selon la revendication 2, dans laquelle le groupe alkyle est le groupe butyle.
- 55 4. Utilisation selon la revendication 2, dans laquelle le groupe alkyle est le groupe hexyle.
5. Utilisation selon la revendication 1, dans laquelle la quantité inhibitrice efficace est comprise entre 50 μ M et 500 μ M.

6. Utilisation selon la revendication 1, dans laquelle le glycolipide est un glycosphingolipide à base de glucocéramide.
7. Utilisation selon la revendication 1, dans laquelle le glycolipide est un glycolipide lysosomal.
- 5 8. Utilisation selon la revendication 1, dans laquelle le glycolipide est un glucocéramide s'accumulant dans les cellules affectées par la maladie de Gaucher.
- 10 9. Utilisation d'un dérivé N-alkyle de désoxygalactonojirimycine dont ledit alkyle contient de 3 à 6 atomes de carbone pour préparer un médicament destiné à inhiber l'expression de surface des récepteurs glycolipidiques aux bactéries et aux toxines bactériennes.
10. Utilisation selon la revendication 9, dans laquelle le groupe alkyle contient de 4 à 6 atomes de carbone.
11. Utilisation selon la revendication 10, dans laquelle le groupe alkyle est un groupe butyle.
- 15 12. Utilisation selon la revendication 10, dans laquelle le groupe alkyle est un groupe hexyle.
13. Utilisation selon la revendication 9, dans laquelle la quantité inhibitrice efficace est comprise entre 50 μ M et 500 μ M.
- 20 14. Utilisation selon la revendication 19, dans laquelle la toxine bactérienne est la toxine cholérique.

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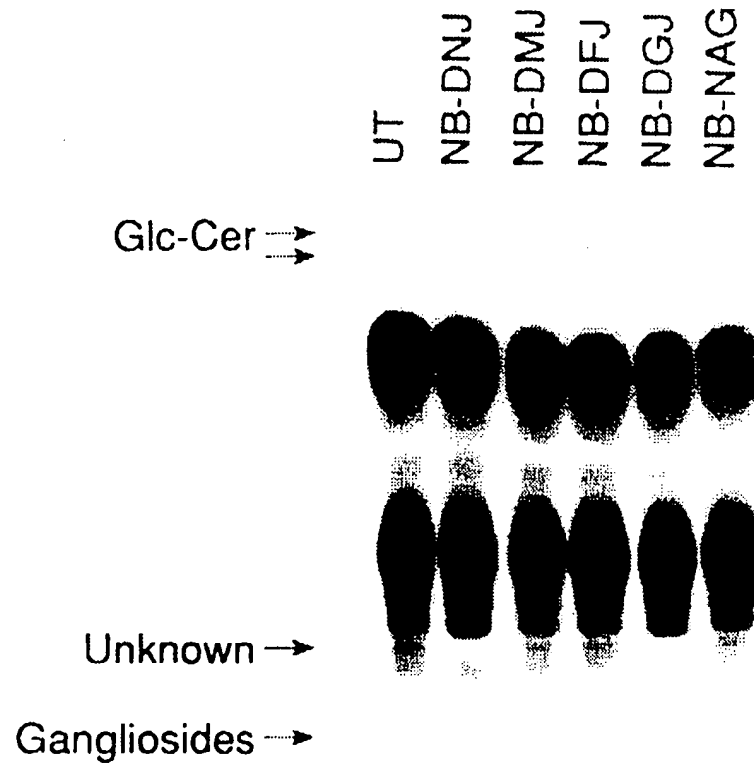


FIG. 1



FIG. 2A

FIG. 2B

FIG. 2C

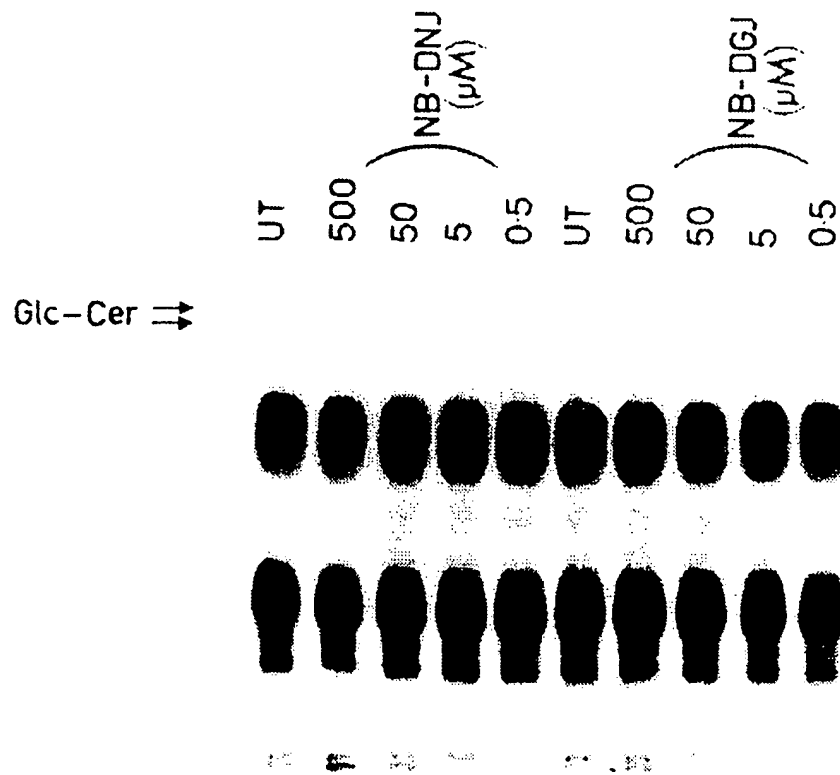


FIG. 3

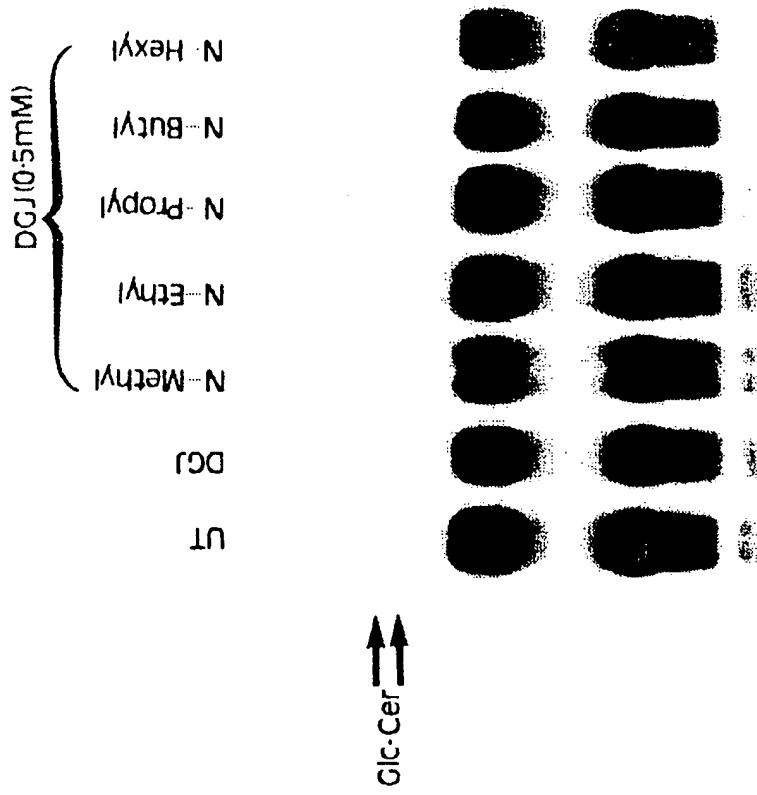


FIG. 4B

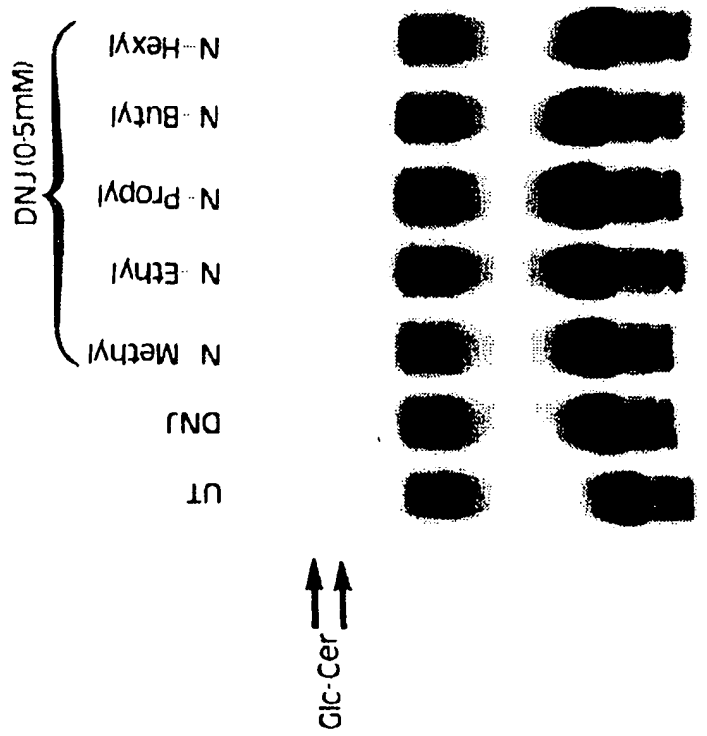


FIG. 4A

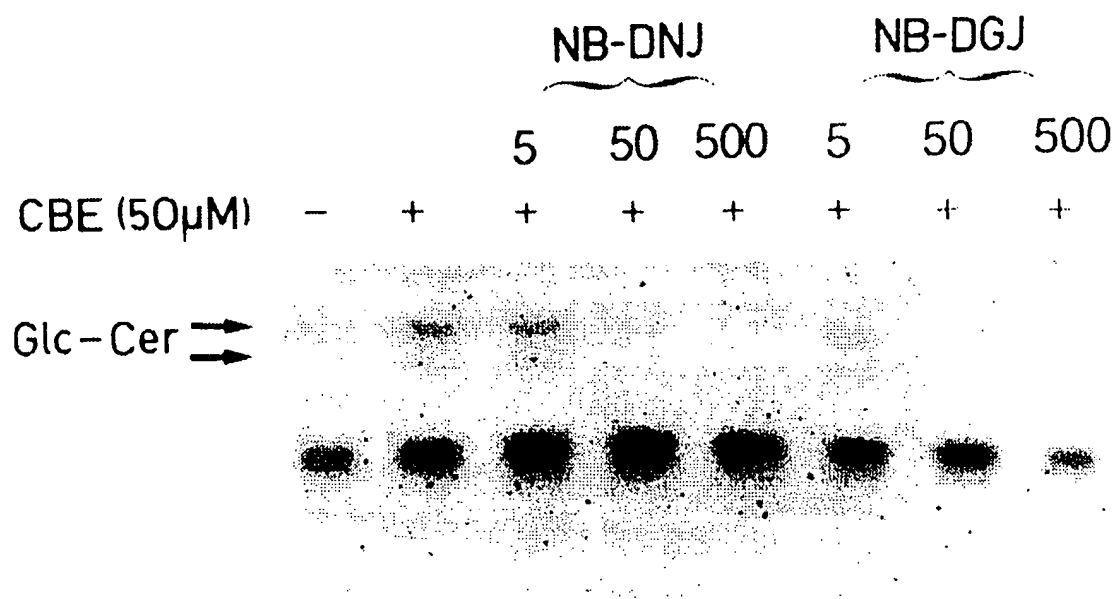


FIG. 5



FIG. 6A



FIG. 6B



FIG. 6C



FIG. 6D



FIG. 6E



FIG. 6F

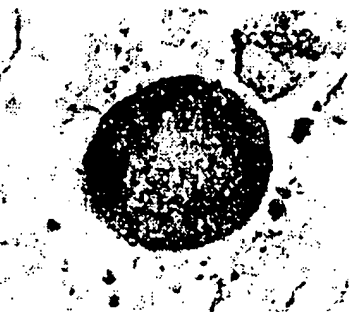


FIG. 6G



FIG. 6H

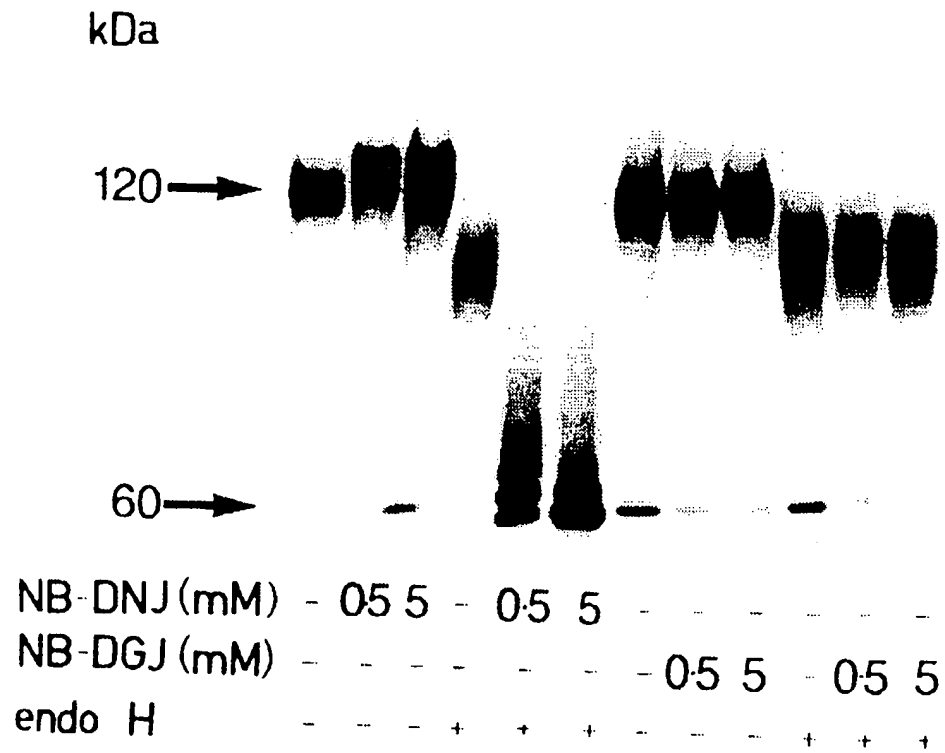


FIG. 7

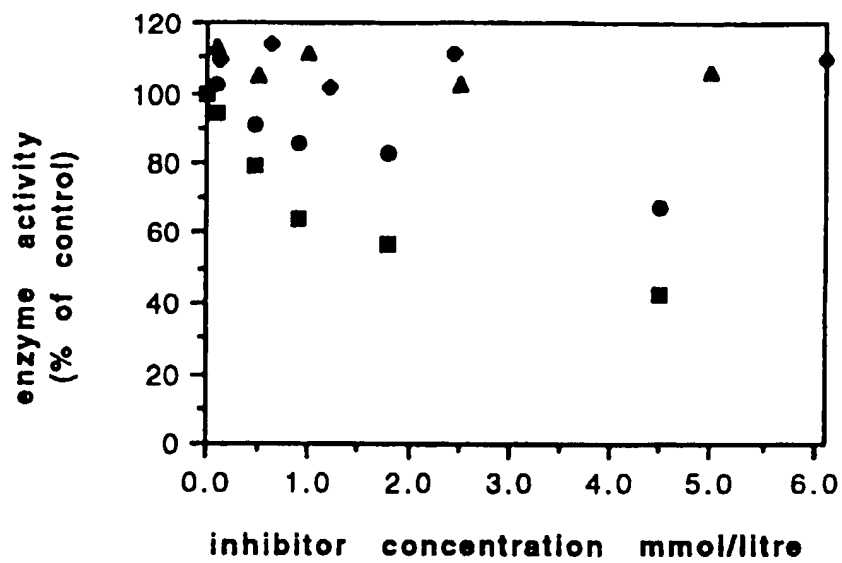


FIG. 8A

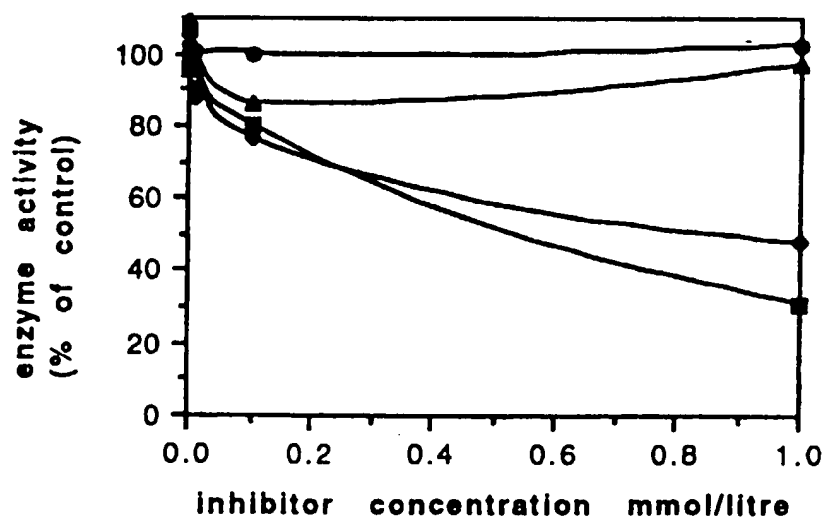


FIG. 8B

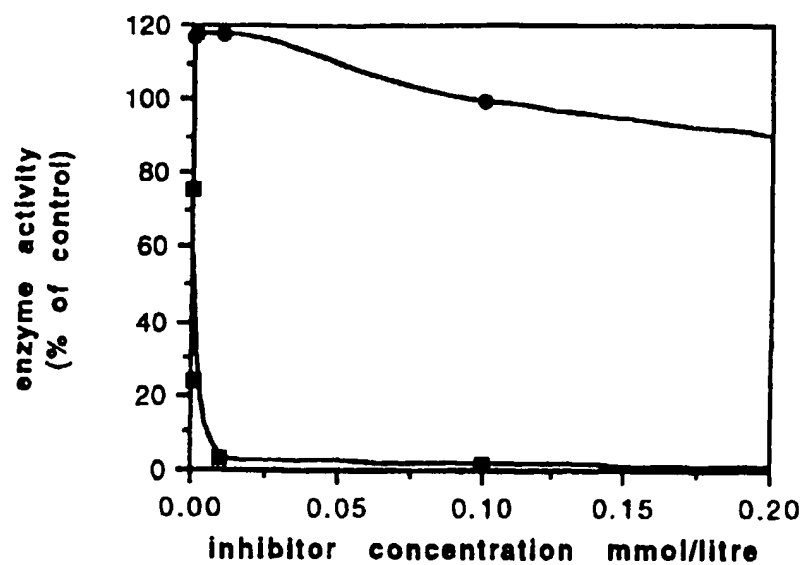


FIG. 8C

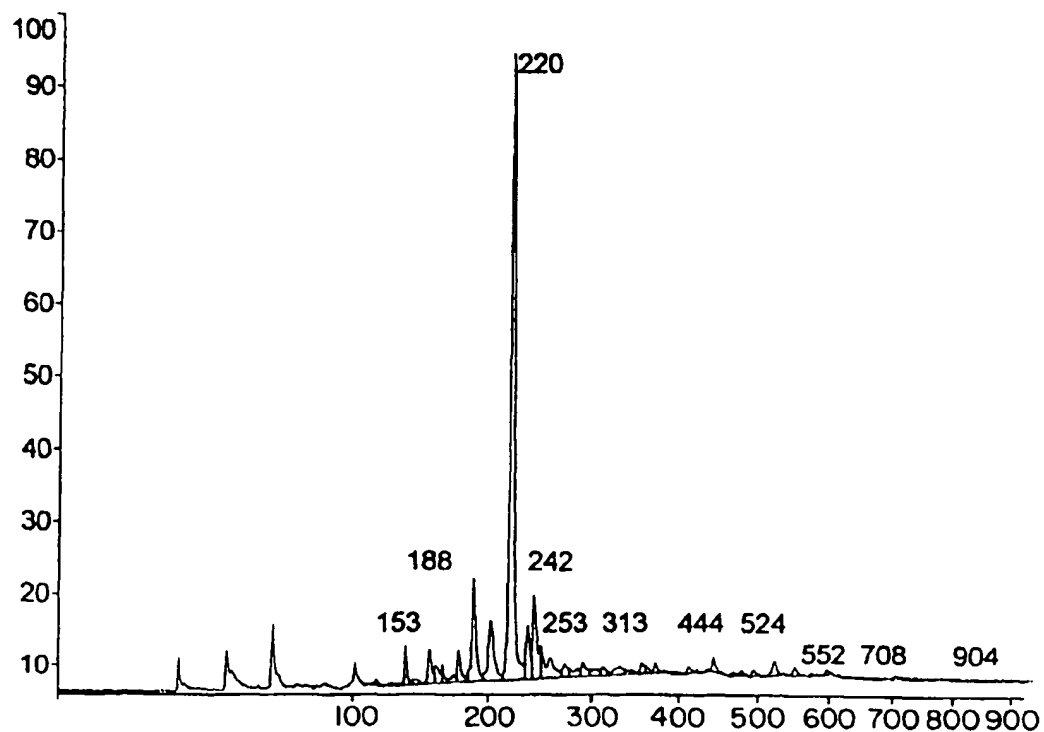


FIG. 9

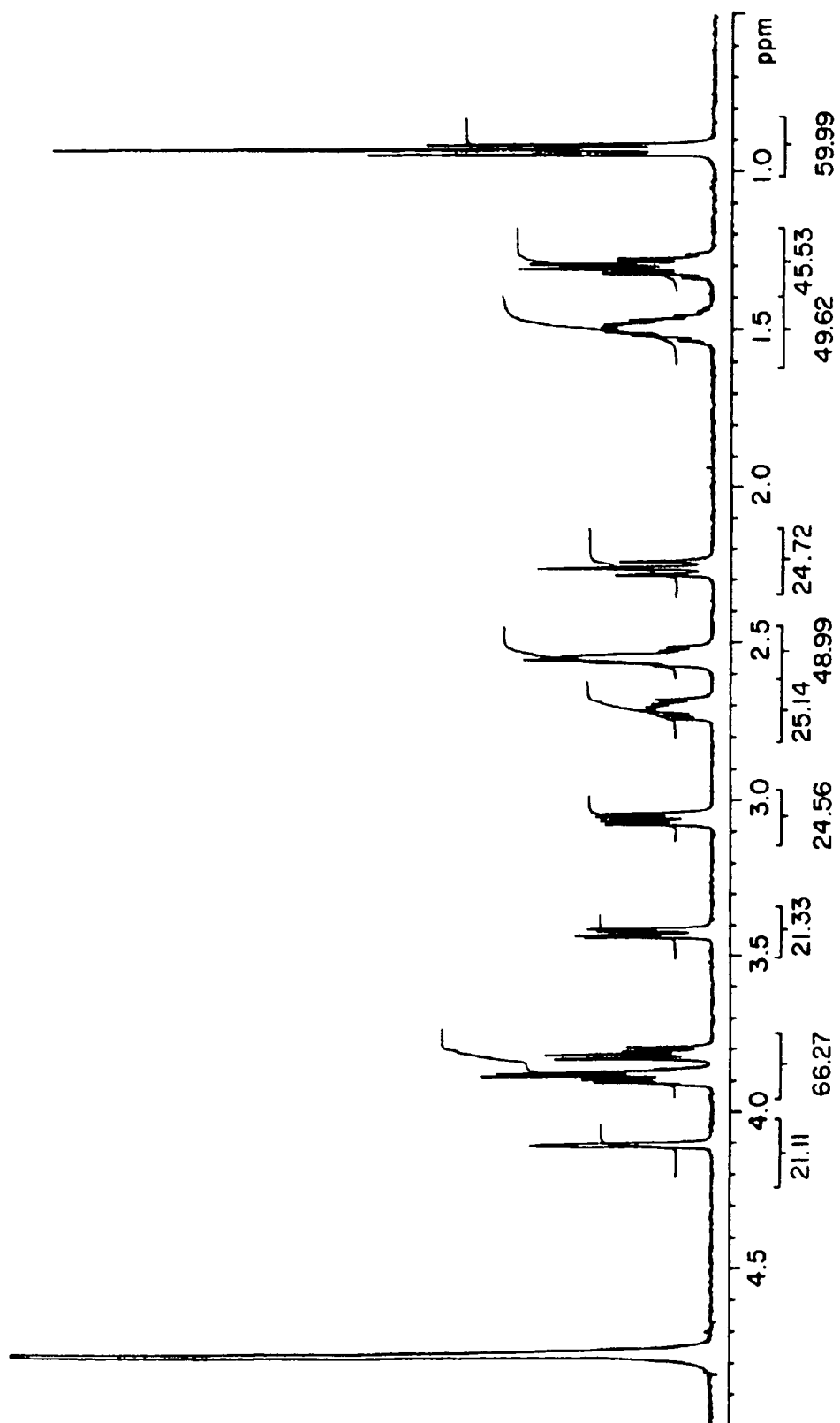


FIG. 10

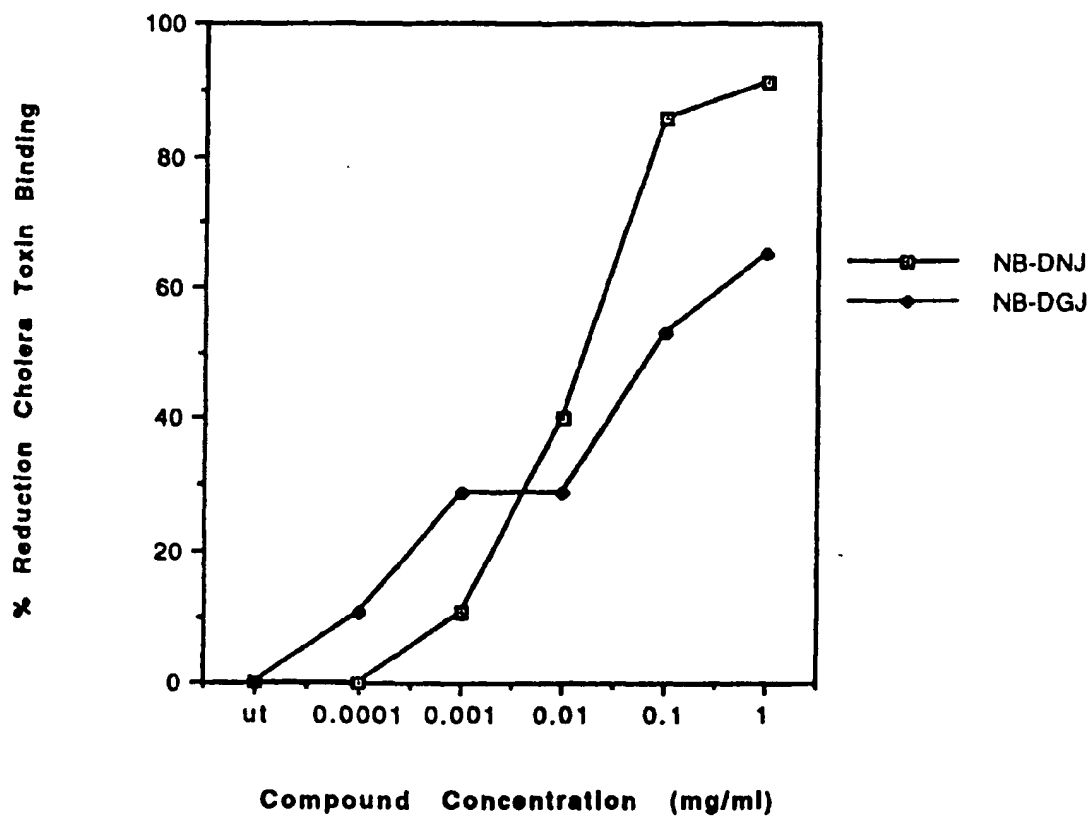
H9 Cells: Cholera Toxin Binding Assay

FIG. 11

NEW N-SUBSTITUTED-1-DEOXYNOJIRIMYCIN DERIVATIVE AND METASTASIS-INHIBITOR FOR CANCEROUS CELL

Publication number: JP2306962 (A)

Publication date: 1990-12-20

Inventor(s): KURIHARA HIROSHI; YOSHIDA SEISHI; TSURUOKA TSUTOMU; TSURUOKA TAKASHI; YAMAMOTO HARUO; FUKUYASU SHUNKAI

Applicant(s): MEIJI SEIKA KAISHA

Classification:

- International: C07D211/46; A61K31/445; A61P35/00; C07D211/00; A61K31/445; A61P35/00; (IPC1-7): A61K31/445; C07D211/46

- European:

Application number: JP19890127499 19890519

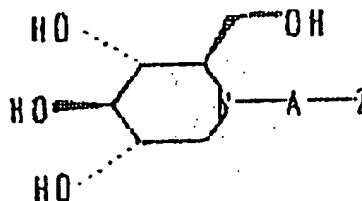
Priority number(s): JP19890127499 19890519

Abstract of JP 2306962 (A)

NEW MATERIAL: An N-substituted-1-deoxynojirimycin derivative expressed by the formula [A is 3-5C hydrocarbon may be substituted with OH, halogenated alkyl or alkoxy (said hydrocarbon may have double or triple bond); Z is phenyl, fluorine-substituted phenyl, biphenyl, cycloalkyl or halogen-substituted alkyl].

EXAMPLE: An N-(3-phenyl-3-trifluoromethyl-2-propenyl)-1-deoxynojirimycin. **USE:** Used as metastasis-inhibitor for cancerous cell.

PREPARATION: For instance, 1-deoxynojirimycin is reacted with various alkylation agent or aralkenylation agent in the presence of deoxidizer such as alkali hydroxide to afford the compound expressed by the formula.



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⑩ 日本国特許庁(JP)

⑪ 特許出願公開

⑫ 公開特許公報(A) 平2-306962

⑬ Int.Cl.⁹

識別記号

庁内整理番号

⑭ 公開 平成2年(1990)12月20日

C 07 D 211/48
A 61 K 31/445

ADU

7180-4C

審査請求 未請求 請求項の数 2 (全12頁)

⑯ 発明の名称 新規N-置換-1-デオキシノジリマイシン誘導体及びそれを含有する癌細胞転移抑制剤

⑰ 特 願 平1-127499

⑱ 出 願 平1(1989)5月19日

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㉑ 代 理 人 弁理士 小堀 益 外1名

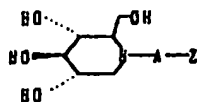
最終頁に続く

明 細 書

1. 発明の名称 新規N-置換-1-デオキシノジリマイシン誘導体及びそれを含有する癌細胞転移抑制剤

2. 特許請求の範囲

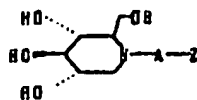
1. 式



式中、Aは水酸基、ハロゲン化アルキル基又はアルコキシ基で置換されてもよい炭素数3乃至5の炭化水素基を表し、この炭化水素基は二重又は三重結合を有していてもよい、Zはフェニル基、フッソ置換フェニル基、ビフェニル基、シクロアルキル基、又はハロゲン置換アルキル基を表す。

で示されるN-置換-1-デオキシノジリマイシン誘導体。

2. 式



式中、Aは水酸基、ハロゲン化アルキル基、アルコキシ基で置換されてもよい炭素数3乃至5の炭化水素基を表し、この炭化水素基は二重又は三重結合を有していてもよい、Zはフェニル基、フッソ置換フェニル基、ビフェニル基、シクロアルキル基又はハロゲン置換アルキル基を表す。

で示されるN-置換-1-デオキシノジリマイシン誘導体又はその薬理的に許容される塩との付加塩を有効成分とすることを特徴とする癌細胞転移抑制剤。

3. 発明の詳細な説明

(産業上の利用分野)

本発明は、癌細胞の転移と形成を阻害する新規N-置換-1-デオキシノジリマイシン誘導体並びにその物質を有効成分とする癌細胞転移抑制剤に関する。

(従来の技術)

現在使用されている抗癌剤は種々あるが、その主体は、癌細胞を殺菌させるか、人の免疫系を

介して死滅させる薬剤であり、癌の根本的な治療に対して有効な薬剤は未だ得られていない。

また、化学療法剤の有効性が低い癌細胞に対しては外科手術、放射線療法等の物理的療法が行われ、原癌の除去という点では成功率が大幅に向上している。しかし、癌細胞の転移を誘発することも事である。

〔発明が解決しようとする課題〕

上述の如く、従来の癌治療において、癌細胞の転移が癌治療患者の予後を左右する最大の問題となっている。

従って、この癌細胞の転移を抑制することが高められる新薬の開発は現在最も要望されている課題である。

本発明はこの課題を解決する癌細胞転移を有効に抑制する物質並びに同物質を有効成分とする癌細胞転移抑制剤を提供することを目的とするものである。

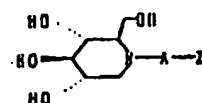
〔課題を解決するための手段〕

本発明者らは先に癌細胞転移抑制作用を有する

N-置換-1-デオキシノジリマイシン誘導体を見出し、特開昭63-31095号公報、特開昭63-93873号公報、特開昭63-97454号公報、特開昭63-104850号公報、特開昭63-147815号公報及び特開昭63-147816号公報に開示した。

本発明者らは更に1-デオキシノジリマイシンの新規なN-置換誘導体を合成し、その広範な評価を行ったところ、強い癌細胞転移抑制作用を有する一群の新規な化合物を見出し、本発明を完成した。

本発明は、式(1)



(1)

(式中、Aは水酸基、ハロゲン化アルキル基又はアルコキシ基で置換されてもよい炭素数3乃至5の炭化水素基を表し、この炭化水素基は二重又は三重結合を有してもよい、Zはフェニル基、フッソ置換フェニル基、ビフェニル基、シクロアルキル基又はハロゲン置換アルキル基を表す、)で示

されるN-置換-1-デオキシノジリマイシン誘導体、並びに同化合物又はその薬理的に許容される酸との付加塩を有効成分とする癌細胞転移抑制剤である。

本発明の式(1)で示されるN-置換-1-デオキシノジリマイシン誘導体は文献未載の新規物質である。

そして、このN-置換-1-デオキシノジリマイシン誘導体に含まれる化合物の例としては次のような物質が挙げられる。

N-(3-メトキシメチル-3-フェニル-2-プロペニル)-1-デオキシノジリマイシン

N-(3-フェニル-3-トリフロロメチル-2-プロペニル)-1-デオキシノジリマイシン

N-(3-(4-フロロフェニル)-2-プロペニル)-1-デオキシノジリマイシン

N-(3-(3-フロロフェニル)-2-プロペニル)-1-デオキシノジリマイシン

N-(3-(2-フロロフェニル)-2-プロペニル)-1-デオキシノジリマイシン

N-(3-(4-ビフェニルプロピル))-1-デオキシノジリマイシン

N-(3-(4-フロロフェニル)-プロピル)-1-デオキシノジリマイシン

N-(3-シクロヘキシルプロピル)-1-デオキシノジリマイシン

N-(3-フェニル-2-プロピニル)-1-デオキシノジリマイシン

N-(2,3-ジヒドロキシ-3-フェニルプロペニル)-1-デオキシノジリマイシン

N-(6,6,8-トリフロロヘキシル)-1-デオキシノジリマイシン

N-(5,5,5-トリフロロペンチル)-1-デオキシノジリマイシン

N-(4,4,4-トリフロロブチル)-1-デオキシノジリマイシン

また、本発明のN-置換-1-デオキシノジリマイシン誘導体を癌細胞転移抑制剤として使用する場合の薬理的に許容される酸の付加塩としては、硫酸、臭化水素酸、硫酸、硝酸、炭酸等の無機酸、

酢酸、酢酸、プロピオン酸、コハク酸、グリコール酸、乳酸、リンゴ酸、酒石酸、クエン酸、マレイン酸、フマル酸、安息香酸、サリチル酸、ノタンズルホン酸等の有機酸、更にはアスパラギン酸、グルタミン酸等のアミノ酸との付加塩が挙げられる。

本発明の化合物はいずれも文献未記載の新規化合物である。その合成法としては本発明者らによって見出された放線菌の代謝産物であるノゾリマイシン (5-アミノ-5-デオキシ-D-グルコピラノース) (特公昭43-760号公報参照) の還元により得られる1-デオキシノゾリマイシン (Tetrahedron, 24, 2125 (1968) 参照) を原料とする方法が最も一般的である。即ち、1-デオキシノゾリマイシンを各種のアルコール類、ジメチルホルムアミド、ジメチルアセトアミド、ジメチルスルホキシド、スルホラン等の極性溶媒又は、それらの混合溶媒中でアラルキルハライド、アルケニルハライド又はアラルキルスルホン酸エステル、アラルケニルスルホン酸エステル等で代表される

各種のアラルキル又はアラルケニル化試料と水酸化アルカリ、炭酸アルカリ、炭酸水素アルカリ又は適当な有機アミン類等の炭酸剤の存在下で室温又は加温することによって本発明の式(1)の化合物のN-置換A-Z基を導入することができる。また、水酸基を適当な保護基、例えばアセチル基、ベンゾイル基、テトラヒドロピラニル基、1-ブチルジメチルシリル基等で保護した1-デオキシノゾリマイシンを原料として用い、N-置換反応を行わたのち、脱保護する方法も採用される。また反応試薬としてカルボニル基を有する試薬を用いて還元的条件下、例えば錯酸、シアノ水素化ホウ素ナトリウム、水素化ホウ素ナトリウム或いは適当な金属触媒、例えば酸化白金、パラジウム、ラネーニッケル等の存在下、水素雰囲気下でいわゆる還元的アルキル化を行う方法、或いは1-デオキシノゾリマイシンとアラルキルカルボン酸、又はアラルケニルカルボン酸とのアミドを還元して目的物を得る方法も使用することができる。これらの化合物は必要に応じて再結晶、カラムクロ

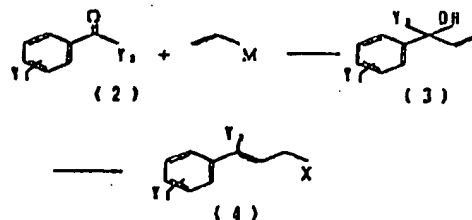
マトグラフィー等の一般的な精製法によって本発明の式(1)の化合物を得る。

本発明の化合物の置換基の形成及び導入に関しては合目的な適宜の方法によって合成することができる。式(1)のA-Z基を調製するためのアラルキル、アラルケニル、アラルケニル化剤の製造については適当な方法として下記の5通りの製造法を示す。

製造法1

化合物(2)とビニル金属化合物、例えば塩化ビニルマグネシウム、臭化ビニルマグネシウム、炭化ビニルマグネシウム、ビニルリチウム、ジビニル亜鉛、ジビニル銅、ジビニルセリウム等とを無活性溶媒中、好ましくはエーテル、テトラヒドロフラン、ジオキサン中で-50℃～室温、10分～24時間反応させることによって化合物(3)を合成することができる。化合物(3)を塩酸、臭化水素酸、オキサリルクロリド、ハロゲン化チオニル、オキシハロゲン化炭、三ハロゲン化炭、五ハロゲン化炭、3置換ホスフィン-四ハロゲン化炭

素、アリル又はアルキルスルホニルハライドと無活性溶媒或いはベンゼン、トルエン、エーテル、塩化メチレン、アセトニトリル等の溶媒中で0℃～100℃、30分～24時間反応させることによって化合物(3)のアリルアルコール部分の経路を伴いながら化合物(4)を合成することができる。



(式中Y₁は水素原子、ハロゲン原子、アラルキル基、水酸基を表し、Y₂は水素原子、ハロゲン原子、アラルキル基、アルコキシ基、ハロゲン置換アルキル基を表す、Xはハロゲン原子、アルキル又はアリルスルホニロキシ基を表す。ハロゲン原子としては、塩素、臭素、炭素等を、アルキル又はアリルスルホニロキシ基としてはノタンズルホニルオキシ基、トリフロロノタンズルホニルオキシ基、

R-トールエンスルホニルオキシ基等を示す。Mは1価又は2価の金属或いはその塩を表し、金属としてはリチウム、ナトリウム、カリウム、マグネシウム、亜鉛、セシウム、銅を示す)

製造法2

化合物(2)を適当な溶媒、好ましくはベンゼン、トルエン、エーテル、テトラヒドロフラン、ジオキサン、塩化メチレン、クロロホルム、メタノール、エタノール中カルボアルコキシメチレントリ置換ホスホランと0℃〜60℃で10分〜24時間反応させるか、又は適当な塩基、例えば水素化ナトリウム、水素化カリウム、水酸化アルカリ、炭酸アルカリの存在下、ジアラルキルホスホノ酢酸アラルキルエステルとを0℃〜60℃で10分〜24時間反応させ、不飽和エステル(5)を合成する。化合物(5)を適当な非プロトン性溶媒、好ましくはエーテル、テトラヒドロフラン、ジオキサン中、適当な水素化金属相体還元剤、好ましくは水素化アルミニウムリチウム、ジイソブチルアルミニウムヒドリド、水素化ビス(2-メトキシエ

キシ)アルミニウムナトリウムと-78℃〜100℃で30分〜18時間反応させることによって化合物(6)を合成することができる。化合物(6)を塩酸、臭化水素酸、オキソリルクロリド、ハロゲン化チオニル、オキシハロゲン化剤、三ハロゲン化剤、五ハロゲン化剤、3置換ホスフィン-四ハロゲン化炭素、アリル又はアルキルスルホニルハライドと無溶媒或いはベンゼン、トルエン、エーテル、塩化メチレン、アセトニトリル等の溶媒中0℃〜100℃で30分〜24時間反応させることにより、化合物(4)を合成することができる。

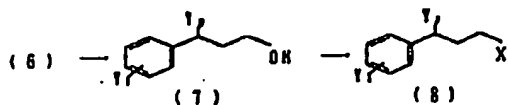


(式中、Y₁、Y₂は前記と同一意味を有し、Rはアルキル基などのカルボキシル基の保護基を表す)

製造法3

製造法2によって得られるアルケニルアルコール(6)を適当な有機溶媒、例えばメタノール、

エタノール、酢酸、テトラヒドロフラン、酢酸エチル等中、金属触媒、例えばパラジウム-炭素、白金、ラネ-ニッケル等の存在下で水素雰囲気下で30分〜24時間還元し、飽和アルコール(7)を合成することができる。化合物(7)を臭化水素酸、オキソリルクロリド、ハロゲン化チオニル、オキシハロゲン化剤、三ハロゲン化剤、五ハロゲン化剤、3置換ホスフィン-四ハロゲン化炭素、アリル又はアルキルスルホニルハライド等の溶媒中で0℃〜100℃、30分〜24時間反応させることにより、化合物(8)を合成することができる。

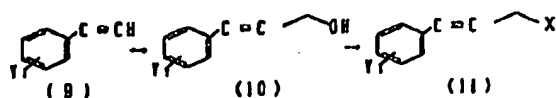


(式中、Y₁、Y₂、Xは前記と同一意味を有す)

製造法4

1-アリルアセチレン誘導体(9)を適当な塩基、例えばn-ブチルリチウム、リチウムジイソプロピルアミド、ナトリウムアミド等でアセチリ

ドとしたのち、ホルマリンと反応させることによって、アルケニルアルコール(10)を合成することができる。化合物(10)をオキソリルクロリド、ハロゲン化チオニル、オキシハロゲン化剤、三ハロゲン化剤、五ハロゲン化剤、3置換ホスフィン-四ハロゲン化炭素、アリル又はアルキルスルホニルハライドと無溶媒或いはベンゼン、トルエン、エーテル、塩化メチレン、アセトニトリル等の溶媒中0℃〜100℃で30分〜24時間反応させることにより、化合物(11)を合成することができる。



(式中Y₁、Y₂、Xは前記と同一意味を有す)

製造法5

末端ハロゲン置換アルキル化剤の製造法としては、例えばω-ハロゲン置換脂肪族(12)を適当なフッ素化剤、例えば四フッ化イオウ(Angew. Chem. International. Ed., 1, 467 (1962))で処理することに

よってトリフロロメチル誘導体(13)を合成することができる。



(式中、Xは前記と同一意味を有す)

以上の製造法1〜5で製造されたアラルキルハライド、アラルケニルハライド又はアラルキルスルホン酸エステル、アラルケニルスルホン酸エステル等で代表される各種のアラルキル又はアラルケニル化試剤と各種アルコール類、ジメチルホルムアミド、ジメチルアセトアミド、ジメチルスルホキシド、スルホラン等の極性溶媒又はそれらの混合溶媒中、水酸化アルカリ、炭酸アルカリ、炭酸水素アルカリ又は、適当な有機アミン類等の炭酸剤の存在下で室温又は加温することによって、本発明の式(1)の化合物のN-置換A-Z基を導入することができる。また、水酸基を適当な保護基、例えばアセチル基、ベンゾイル基、テトラヒ

(式中、Y₁、Y₂は前記と同一意味を有す、R'は水素原子、アセチル基、ベンゾイル基、ベンゾイル基、ビバロイル基、1-ブチルジメチルシリル基、テトラヒドロピラニル基を示す)

次に本発明のN-置換-1-デオキシノジリマイシン誘導体の製造例を示す。

製造例1

N-(3-フェニル-3-トリフロロメチル-2-プロペニル)-1-デオキシノジリマイシン
工程1

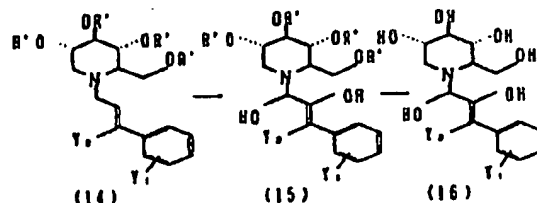
3-フェニル-3-トリフロロメチル-2-プロペニル-1-オール

2, 2, 2-トリフロロアセトフェノン1.76g (10.0 mmol) をテトラヒドロフラン10mlに溶かした溶液を-78℃に冷却し、1Mビニルマグネシウムブロミドテトラヒドロフラン溶液を加える。加え終了後3時間同温度で攪拌後、冷却を取り去り1時間攪拌する。氷冷水を加えて過剰の試薬を分解した後、溶液を留去する。残液に2N炭酸10ml加え、酢酸エチルで抽出する。抽出液を

ドロピラニル基、1-ブチルジメチルシリル基等で保護した1-デオキシノジリマイシンを原料として用い、N-置換反応を行わせた後、脱保護する方法も採用される。本発明に含まれる化合物のうち、式(1)中Aが水酸基で置換された炭化水素であるものについては、次に示す製造方法6によって製造することができる。

製造法6

製造法1、或いは2に従って合成したアルケニル化剤と1-デオキシノジリマイシン或いは水酸基を保護した1-デオキシノジリマイシンとを反応させることによって合成することができるN-置換-1-デオキシノジリマイシン誘導体(14)を適当な酸化剤、例えば四酸化オスミウム等と反応させ目的物(16)を得ることができる。



水洗、乾燥後濃縮する。残渣をシリカゲルカラムクロマトグラフィー（溶出溶媒：エーテル-ヘキサン(1:10)）で精製し、1.66g(82%)の抽出物を得た。

¹HMR(CDCl₃) δ

2.61(s, 1H), 5.52(d, 1H), 5.62(d, 1H),

6.43(dd, 1H), 7.25 ~ 7.70(m, 5H)

工程2

1-ブチル-3-フェニル-3-トリフロロメチル-2-プロペン

3-フェニル-3-トリフロロメチル-2-プロペン-1-オール606 mg(3.00 mmol) とトリフェニルホスフィン943 mg(3.60 mmol) をアセトニトリル4mlに溶解し氷冷する。ここへ四酸化オスミウム1.26g(3.80 mmol)を数回に分けて加える。氷冷下1時間攪拌した後、一夜室温下攪拌する。反応液をエーテル10mlで希釈し、析出する固体を濾過し、母液を濃縮する。得られる残渣をシリカゲルカラムクロマトグラフィー（溶出溶媒：ヘキサン）で精製し、440 mg(55%)の抽出物

を得た。

$\text{NMR}(\text{CDCl}_3)$ δ

3.80(dq, 2H), 6.62(tq, 1H), 7.20~7.60(m, 5H)

工程 3

N-(3-フェニル-3-トリフロロメチル-2-プロペニル)-1-デオキシノジリマイシン
デオキシノジリマイシン163 mg(1.00 mmol)と1-ブロモ-3-フェニル-3-トリフロロメチル-2-プロペン318 mg(1.20 mmol)をジメチルホルムアミド5 mlに溶解し、炭酸カリウム207 mg(1.50 mmol)を加えて室温下8時間攪拌する。反応混合物に飽和食塩水を加えてn-ブタノールで抽出する。抽出液を減圧下濃縮し、残液をシリカゲルカラムクロマトグラフィー(抽出溶媒:クロロホルム-nブタノール(10:1))で精製し311 mg(90%)の無色固体を得た。

$\text{NMR}(\text{CD}_3\text{OD})$ δ

2.15(m, 2H), 3.10(dd, 1H), 3.16(t, 1H),

3.31(m, 1H), 3.42(t, 1H), 3.53(m, 1H),

3.78(dd, 1H), 3.98(ABX type, 2H).

メル)を塩化ノチレン20 mlに溶解し、カルボノトキシメチレントリフェニルホスホラン3.67 g(11.0 mmol)を加え、室温下3時間攪拌した。固体を濾別し、母液を濃縮し、残液をシリカゲルカラムクロマトグラフィー(抽出溶媒:酢酸エチル-ヘキサン(1:4))で精製し、無色針状品1.81 g(90%)を得た。

$\text{NMR}(\text{CDCl}_3)$ δ

4.30(d, 2H), 6.25(m, 1H), 6.55(d, 1H),

6.95(m, 2H), 7.35(m, 2H)

工程 2

3-(4-フロロフェニル)-2-プロペン-1-オール

メチル-3-(4-フロロフェニル)-2-プロペノエート1.61 g(9.00 mmol)をエーテル50 mlに溶解し、氷冷下水素化アルミニウムリチウム205 mg(5.40 mmol)をエーテル3 mlに懸濁したものに加下する。減圧下室温下30分攪拌し、過剰の試薬を水で分解し、固体を濾別する。母液を濃縮し3-(4-フロロフェニル)-2-プロ

6.72(t, 1H), 7.32(m, 2H), 7.46(m, 3H)

製造例 2

N-(3-メトキシメチル-3-フェニル-2-プロペニル)-1-デオキシノジリマイシン

製造例1と同様にして合成した1-ブロモ-3-メトキシメチル-3-フェニル-2-プロペンを用いて合成した。

$\text{NMR}(\text{CD}_3\text{OD})$ δ

2.13(m, 2H), 3.06(dd, 1H), 3.16(t, 1H),

3.34(m, 1H), 3.44(t, 1H), 3.31(m, 1H),

3.38(s, 3H), 3.76(dd, 1H),

3.97(ABX type, 2H), 4.16(s, 2H),

6.08(t, 1H), 7.15~7.50(m, 5H)

製造例 3

N-(3-(4-フロロフェニル)-2-プロペニル)-1-デオキシノジリマイシン

工程 1

メチル-3-(4-フロロフェニル)-2-プロペノエート

4-フロロベンズアルデヒド1.24 g(10.0 mmol)

ベン-1-オール1.33 g(97%)を得た。

$\text{NMR}(\text{CDCl}_3)$ δ

4.52(d, 2H), 6.31(m, 1H), 7.01(m, 2H),

7.45(m, 2H)

工程 3

1-ブロモ-3-(4-フロロフェニル)-2-プロペン

3-(4-フロロフェニル)-2-プロペン-1-オール1.34 g(8.82 mmol)とトリ-n-オクタチルホスフィン4.28 g(11.5 mmol)をエーテル20 mlに溶解し、氷冷下四氯化炭3.52 g(10.6 mmol)を数回に分け加える。室温下30分攪拌した後、沈殿物を濾別し、母液を濃縮し残液をシリカゲルカラムクロマトグラフィー(抽出溶媒:ヘキサン)で精製し1.61 g(85%)の無色結状物を得た。

$\text{NMR}(\text{CDCl}_3)$ δ

3.35(d, 2H), 6.30(m, 1H), 7.00(m, 2H),

7.40(m, 2H)

Mass m/z 214, 216

工程4

N-(3-(4-フロロフェニル)-2-プロベニル)-1-デオキシノジリマイシン

1-プロモ-3-(4-フロロフェニル)-2-プロペン1.61g (7.5ミリモル)と1-デオキシノジリマイシン1.22g (7.5ミリモル)をジノチルホルムアミド10mlに溶解し、炭酸カリウム3.12g (22.5ミリモル)を加え、室温下24時間攪拌した。反応混合物を水に注いでn-ブタノールで抽出する。溶液を留去した後、残渣をシリカゲルカラムクロマトグラフィー(抽出溶媒:クロロホルム-メタノール(10:1))で精製し1.36g (61%)の淡黄色の固体を得た。

¹HMR(CD₃COO) δ

2.4~4.2(m, 16H), 6.40(m, 1H), 6.7(m, 1H), 7.10(m, 2H), 7.55(m, 2H)

Mass m/z 298 (PD, M+1)

製造例4

N-(3-(3-フロロフェニル)-2-プロベニル)-1-デオキシノジリマイシン

Mass m/z (PD, M+1)

製造例6

N-(3-(4-ビフェニル)プロピル)-1-デオキシノジリマイシン

工程1

メチル-3-(4-ビフェニル)アクリレート4-ビフェニルカルボキシアルデヒド1.10g (5.00ミリモル)をジクロロエタン20mlに溶解し、カルボメトキシメチレントリフェニルホスホラン3.03g (9.10ミリモル)を加え、室温下1時間攪拌する。溶液を留去後、残渣をシリカゲルカラムクロマトグラフィー(抽出溶媒:エーテル-ヘキサン(1:10))で精製し、1.12g (78%)の無色結晶を得た。

¹HMR(CDCl₃) δ

3.83(s, 3H), 6.49(d, 1H), 7.30~7.60(m, 9H), 7.75(d, 1H)

工程2

メチル-3-(4-ビフェニル)プロピオネート

製造例3と同様にして合成した。

¹HMR(CD₃COO) δ

2.15(m, 2H), 3.04(dd, 1H), 3.14(t, 1H), 3.2~3.35(m, 1H), 3.39(t, 1H), 3.49(m, 1H), 3.68(dd, 1H), 3.94(ABX type, 2H), 6.41(d, 1H), 6.59(d, 1H), 6.95(d, 1H), 7.16(dd, 1H), 7.21(d, 1H), 7.31(ddd, 1H)

Mass m/z 298 (PD, M+1)

製造例5

N-(3-(2-フロロフェニル)-2-プロベニル)-1-デオキシノジリマイシン

製造例3と同様にして合成した。

¹HMR(CD₃COO) δ

2.1~2.25(m, 2H), 3.06(dd, 1H), 3.14(t, 1H), 3.24~3.35(m, 1H), 3.39(t, 1H), 3.50(m, 1H), 3.71(m, 1H), 3.94(ABX type, 2H), 6.45(d, 1H), 6.72(d, 1H), 7.0~7.16(m, 2H), 7.2~7.28(m, 1H), 7.53(d, 1H)

メチル-3-(4-ビフェニル)アクリレート1.40g (4.40ミリモル)を酢酸エチル50mlに溶解し、10%Pd-C70mgを加えて常圧下12時間攪拌還元する。溶液を蒸留後、溶液を留去し、1.01g (97%)の無色結晶物を得た。

¹HMR(CDCl₃) δ

2.68(t, 2H), 3.00(t, 2H), 3.68(s, 3H), 7.20~7.70(m, 9H)

工程3

3'-(4-ビフェニル)-1-プロパロール氷冷下、水酸化アルミニウムリチウム110mg (2.90ミリモル)をエーテル10mlに懸濁した中へメチル-3-(4-ビフェニル)プロピオネート1.01g (4.20ミリモル)をエーテル35mlに溶解したものを滴下する。同温度で1時間攪拌後、過剰の試薬を水で分解し、無機物を蒸留、残渣を乾燥後、蒸留し、861mg (96%)の無色結晶を得た。

¹HMR(CDCl₃) δ

1.56(br, 1H), 1.94(m, 2H), 2.77(m, 2H), 3.71(m, 2H), 7.15~7.76(m, 9H)

工程4

3-(4-ビフェニル)-1-ブロモプロパン
3-(4-ビフェニル)-1-プロパノール
119 g (2.00ミリモル) とトリフェニルホスフィン
629 g (2.40ミリモル) をエーテル10mlに溶解し
氷冷下四臭化炭素 930 g (2.80ミリモル) を攪
拌に分け加える。室温下1時間攪拌した後、反応
物を蒸留し、残渣を濃縮し残渣をシリカゲルカ
ラムクロマトグラフィー(溶出溶媒:ヘキサン)で
精製し506 g (92%) の無色油状物を得た。

NMR(CDCl₃) δ

2.20(quin, 2H), 2.83(t, 2H), 3.44(t, 2H),
7.23~7.65(m, 9H)

工程5

N-(3-(4-ビフェニル)プロピル)-1-
デオキシノジリマイシン

3-(4-ビフェニル)-1-ブロモプロパン
140 g (0.50ミリモル) と1-デオキシノジリ
マイシン82 g (0.5ミリモル) をジメチルホルム
アミド1mlに溶解し、炭酸カリウム136 g (1.00

製造例8

N-(3-シクロヘキシルプロピル)-1-
デオキシノジリマイシン

製造例6と同様に合成した。

NMR(CDCl₃) δ

0.75~1.08(m, 2H), 1.08~1.45(m, 1H),
1.45~2.00(m, 8H), 2.70~3.83(m, 8H),
4.00(ABX type, 2H)

製造例9

N-(フェニル-2-プロピニル)-1-
デオキシノジリマイシン

工程1

1-フェニル-3-プロモプロピン

1-フェニル-2-プロピン-1-オール660
g (5.00ミリモル) と四臭化炭素4.98 g (15.0
ミリモル) をテトラヒドロフラン30mlに溶解し、氷
冷下トリフェニルホスフィン2.62 g (10.0ミリ
モル) を攪拌に分けて加える。室温下10時間攪拌後、
固体を蒸留し、残渣を濃縮する。残渣をシリカゲ
ルカラムクロマトグラフィー(溶出溶媒:ヘキサ

ン)を加え、80℃、4時間加熱した。反応混
合物を水に注いで塩酸酸性としエーテルにて洗浄。
水層をアンモニアアルカリとし、n-ブタノール
で抽出する。残渣を除去した後、残渣をシリカゲ
ルカラムクロマトグラフィー(溶出溶媒:クロ
ホルム-ノタノール(10:1))で精製し117 g
(66%) の固体を得た。

NMR(CDCl₃) δ

1.86(m, 2H), 2.20(br, 2H), 2.65(m, 3H),
2.89(m, 1H), 3.00(m, 1H), 3.14(t, 1H),
3.47(m, 1H), 3.84(d, 2H), 7.15~7.65(m, 9H)

製造例7

N-(3-(4-フロロフェニル)プロピル)-
1-デオキシノジリマイシン

製造例6と同様に合成した。

NMR(CDCl₃) δ

1.38(m, 2H), 2.05~2.22(m, 2H), 2.64(m, 2H),
2.98(dd, 1H), 3.13(t, 1H), 3.30(m, 1H),
3.38(t, 1H), 3.45(m, 1H),
3.64(m, 1H), 3.85(m, 2H), 7.18~7.35(m, 4H)

ン)で精製し、181 g (65%) の無色油状物を得
た。

NMR(CDCl₃) δ

1.20 (br, 1H), 2.27(m, 1H), 7.15~7.40(m, 5H)

工程2

N-(フェニル-2-プロピニル)-1-
デオキシノジリマイシン

1-デオキシノジリマイシン163 g (1.00ミリ
モル) と1-フェニル-3-プロモプロピン215
g (1.10ミリモル) をジメチルホルムアミド3ml
に溶解し、炭酸カリウム166 g (1.20ミリモル)
を加え、室温下8時間攪拌する。反応混合物を水
に注いで塩酸酸性としエーテルにて洗浄、水層を
アンモニアアルカリとし、n-ブタノールで抽出
する。残渣を除去した後、残渣をシリカゲルカ
ラムクロマトグラフィー(溶出溶媒:クロホル
ム-ノタノール(10:1))で精製し、181 g (65
%) の固体を得た。

NMR(CDCl₃) δ

2.31(d, 1H), 2.57(t, 1H), 2.98(dd, 1H),

3.19(t, 1H), 3.50(t, 1H), 3.61(o, 1H),
3.82(ABX type, 2H), 3.98(dd, 2H)

製造例10

N-(2, 3-ジヒドロキシ)-3-フェニ
ルプロピル)-1-デオキシノジリマイシン

工程1

N-(3-フェニル-2-プロペニル)-1-
デオキシノジリマイシンテトラアセテート

シンナミルプロピル 1.42 g (7.20ミリモル) と
1-デオキシノジリマイシン 978 mg (6.00ミリモ
ル) をジメチルホルムアミド 10 ml に溶解し、炭酸
カリウム 996 mg (7.20ミリモル) を加えて、4時
間、60~65℃に加熱する。冷却、塩化メチレン 3
ml で希釈し、無水酢酸 3.06 g (30.0ミリモル) と
ピリジン 2.37 g (30.0ミリモル) を加えて室温下
16時間攪拌する。反応液を酢酸エチル 150 ml で希
釈し、飽和炭酸水素ナトリウム、水で順次洗浄、
乾燥後、溶媒を留去する。残液をシリカゲルカラ
ムクロマトグラフィー（溶出溶媒：ヘキサン-酢
酸エチル（3：1））で精製し、2.12 g (81%)

収：ヘキサン-酢酸エチル（1：1）で精製し、
222 mg (68%) のカラメルを得た。この化合物は
2種の立体異性体の混合物（2：1）である。

¹HMR(CDCl₃) δ

2.32(dd), 2.57(dd), 2.70(ABX type), 2.85(dd),
2.97(m), 3.11(m), 3.12(dd), 3.16(s), 3.22(dd),
3.82(br), 4.13(ABX type), 4.20(ABX type),
4.48(t), 4.53(t), 4.86~5.12(m),
7.2 ~ 7.4(m, 5H)

工程3

N-(2, 3-ジヒドロキシ)-3-フェニ
ルプロピル)-1-デオキシノジリマイシン

N-(2, 3-ジヒドロキシ)-3-フェニ
ルプロピル)-1-デオキシノジリマイシンテ
トラアセテート 196 mg (0.42ミリモル) をメタノ
ール 5 ml に溶解し、炭酸カリウム 3 mg を加えて室温
下 3時間攪拌する。溶媒を留去した後、残液をシ
リカゲルカラムクロマトグラフィー（溶出溶媒：
クロロホルム-メタノール（3：1））で精製し
128 mg (98%) の無色カラメルを得た。この化合

物の結晶を得た。

¹HMR(CDCl₃) δ

2.01(s, 6H), 2.03(s, 3H), 2.09(s, 3H),
2.38(dd, 1H), 2.70(dt, 1H), 3.25(dd, 1H),
3.38(dd, 1H), 3.59(ddd, 1H), 4.19(dd, 1H),
4.32(dd, 1H), 4.90~5.20(m, 3H), 5.22(dt, 1H),
6.56(d, 1H), 7.15 ~ 7.50(m, 5H)

工程2

N-(2, 3-ジヒドロキシ)-3-フェニ
ルプロピル)-1-デオキシノジリマイシンテ
トラアセテート

N-(3-フェニル-2-プロペニル)-1-
デオキシノジリマイシンテトラアセテート 305 mg
(0.70ミリモル) と N-メチルモルホリン-N-
オキシド 98 mg (0.84ミリモル) を 50% アセトン 8
ml に溶解し、四酸化オスミウム 2 mg を加え 2時間
攪拌する。亜硫酸ナトリウム 250 mg、水 3 ml を加
えて 1時間攪拌した後、水 30 ml で希釈し酢酸エチ
ルで抽出、水洗、乾燥後、溶媒を留去する。残液
をシリカゲルカラムクロマトグラフィー（溶出溶

物は 2種の立体異性体の混合物（2：1）である。

¹HMR(CD₃COO) δ

2.05(dd), 2.17(dd), 2.23~2.35(m), 2.54(dd),
2.87(dd), 2.98(dd), 3.10(t), 3.14(t),
3.2 ~ 4.0(m), 4.50(d), 4.68(d),
7.15~7.50(m, 5H).

次に本発明の N-置換-デオキシノジリマイシ
ン誘導体の癌細胞転移抑制作用の評価結果を示す。
効果試験

試験法

マウスの腫瘍細胞であるメラノーマ B16 株より
フィドラー (Fidler) の方法 (Method in Cancer
Research, 15, 339-439, 1978) をもとに B16 高転
移株を選別し、使用した。転移抑制作用の評価は
キジマ-スダ (Kijima-Suda) 等の方法 (Proc.,
Natl. Acad. Sci., U.S.A., 83, 1752-1756,
1986; Cancer Research, 46, 858-862, 1986.) を
もとにして行った。まず B16 高転移株を牛胎児血
清を加えたダルベコ ME 培地 (DMEM 培地) に植
え、一般式 (1) で表される N-置換-1-デオ

キシノグリマイシンを加え、2～4日間、5%CO₂の存在下37℃で培養し、増殖した細胞をトリプシン-EDTA溶液で培養容器より剥がした。この細胞をCa⁺⁺とMg⁺⁺を含まないダルベコの平衡塩溶液で生細胞として1ml当たり1×10⁶細胞になるように懸濁した。

この懸濁液の0.1 mlをマウス尾静脈中に注入し細胞を移植し14日間飼育した後、開腹して肺を摘出し、肺表面及び内部に形成されたB16高転移株の転移結節数を数え、薬剤処理をしなかった対照と比較した。

試験例1 細胞障害性

B16高転移株を10%牛胎児血清を加えたDMEM培地で5%CO₂の存在下37℃で培養し、トリプシン-EDTA溶液で培養容器より剥がし、1ml当たり1×10⁶細胞になるように懸濁した。この懸濁液の150 μlを被検薬あるいは対照薬溶液50 μlにそれぞれ加え混合した。この後、4日間培養し、倒立顕微鏡下で生死を観察し、細胞障害性を判定した。その結果を表1の通りであった。

表 1		
使用細胞	B16高転移株	
添加薬剤	濃度	生育
無添加		+
製造例化合物 9	10 μg/ml	+
	30 μg/ml	+
	100 μg/ml	+
製造例化合物 10	10 μg/ml	+
	30 μg/ml	+
	10 μg/ml	+
製造例化合物 7	10 μg/ml	+
	30 μg/ml	+
	100 μg/ml	+
アドリアマイシン (対照)	0.1 μg/ml	-

表中+は生育、-は死滅を意味す。

以上の試験結果より本発明の化合物はB16高転移株に対して細胞障害性を示さなかった。

試験例2 抗転移作用

B16高転移株を10%牛胎児血清を加えたDMEM培地に植え、被検薬を1ml当たりそれぞれ30 μg加え、5%CO₂の存在下37℃で3日間培養した。試験例1と同様の方法で細胞を培養容器より剥がした。この細胞をCa⁺⁺とMg⁺⁺を含まないダルベコ

の平衡塩溶液で生細胞として1ml当たり1×10⁶細胞になるように懸濁し、その0.1 mlをBDF₁マウス(8週令、雄)の尾静脈に注入し、細胞を移植した。14日間飼育後、開腹して肺を摘出し、肺表面及び内部に形成されたB16高転移株の転移結節数を数えた。その結果を表2に示した。

表 2

添加薬剤	肺転移結節数 (平均±標準偏差)
無添加	207±47
製造例化合物 9 (30 μg/ml)	96±29
製造例化合物 10 (30 μg/ml)	60±18
製造例化合物 7 (30 μg/ml)	18±7

以上の結果より本発明の化合物の処理で肺に形成される転移結節数は大きく減少した。

本発明の癌細胞転移阻害剤は、上記のN-置換-1-デオキシノグリマイシン誘導体を含有する経口、非経口製剤とし臨床的に肺癌、肺癌、皮膚癌、皮下、皮内、直腸及び筋肉内を経由又は経口にて投与される。また腫瘍に直接投与することにより、より強い効果が見待できる。投与量は投与形態、

剤型あるいは患者の年齢、体重、病態により異なるが、概ね1日100～3000mgを1回又は数回投与する。

非経口製剤としては、薬液の水溶性又は非水性溶媒剤あるいは乳剤が挙げられる。非水性の溶媒剤又は乳剤の溶媒としては、プロピレングリコール、ポリエチレングリコール、グリセリン、オリーブ油、とうもろこし油、オレイン酸エステル等が挙げられる。

また、経口剤としては、カプセル剤、錠剤、咀嚼剤、散剤等が挙げられる。

これらの製剤に賦形剤として、澱粉、乳糖、マンニト、エチルセルロース、ナトリウムカルボキシメチルセルロース等が配合され、滑沢剤としてステアリン酸マグネシウム又はステアリン酸カルシウムを添加する。結合剤としては、ゼラチン、アラビアゴム、セルロースエステル、ポリビニルピロリドン等が用いられる。

次に本発明の製剤例について説明する。

(実施例)

である。

N-(3-(4-フロロフェニル)-2-プロペニル)-1-

デオキシノジリマイシン 200 mg

乳糖 130 mg

ジャガイモ澱粉 70 mg

ポリビニルピロリドン 10 mg

ステアリン酸マグネシウム 2.5 mg

乳糖及びジャガイモ澱粉を混合し、これにポリビニルピロリドンの20%エタノール溶液を加え、均一に遊離させ、100の篩目のふるいを通し、45℃にて乾燥させ、再度100の篩目のふるいを通した。こうして得られた顆粒をステアリン酸マグネシウムと混合し錠剤に成型した。

(発明の効果)

本発明は癌細胞転移抑制作用を有する極めて有用な物質である。そして、この物質を有効成分とした癌細胞転移抑制剤は、現在この防止手段が殆ど無く、癌治療患者の予後を左右する最大の問題である癌細胞の転移を解決した極めて有用な発明

特許出願人

明治製菓株式会社

代理人

小 関 啓 (ほか1名)

第1頁の続き

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手 続 補 正 書

平成元年10月27日

特許庁長官 宮 田 文 毅 殿

1. 事件の表示

平成1年 特 許 願 第127499号

2. 発明の名称 新規N-置換-1-デオキシノジリマイシン

誘導体及びそれを含有する癌細胞転移抑制剤

3. 補正をする者

事件との関係 特許出願人

氏 名 (509) 明治製菓株式会社

4. 代理人

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氏 名 (8216) 弁理士 小 堀 益

5. 補正の対象

明細書

6. 補正の内容

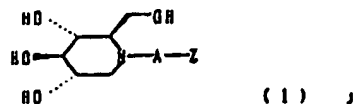


方式 ④

5の炭化水素基を表し、この炭化水素基は二重又は三重結合を有していてもよい、Zはフェニル基、フッソ置換フェニル基、ピフェニル基、シクロアルキル基又はハロゲン置換アルキル基を表す。

で示されるN-置換-1-デオキシノジリマイシン誘導体又はその薬理的に許容される鹽との付加塩を有効成分とすることを特徴とする癌細胞転移抑制剤。」

(2) 明細書第4頁の式(1)を下記の通り補正する。



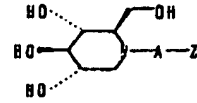
(3) 明細書第3頁第12～14行「従って、この・・・」を下記の通り補正する。

「従って、現行の癌治療の有効性は癌細胞の転移を抑制することで、さらに高められることが期待される。」

(4) 明細書第15頁下から第9行「ケニル化試剤と

(1) 特許請求の範囲を下記の通り補正する。

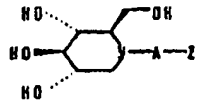
1. 式



式中、Aは水酸基、ハロゲン化アルキル基又はアルコキシ基で置換されてもよい炭素数3乃至5の炭化水素基を表し、この炭化水素基は二重又は三重結合を有していてもよい、Zはフェニル基、フッソ置換フェニル基、ピフェニル基、シクロアルキル基、又はハロゲン置換アルキル基を表す。

で示されるN-置換-1-デオキシノジリマイシン誘導体。

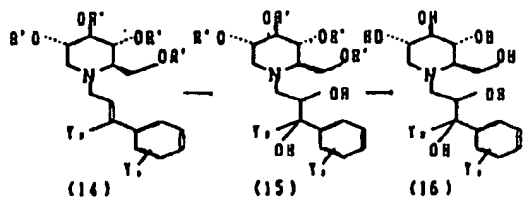
2. 式



式中、Aは水酸基、ハロゲン化アルキル基、アルコキシ基で置換されてもよい炭素数3乃至

各種アルコール類」を「ケニル化試剤と1-デオキシノジリマイシンを各種アルコール類」に補正する。

(5) 明細書第16頁の式(14)、(15)、(16)をそれぞれ下記の通り補正する。



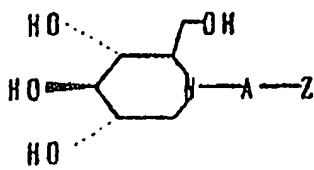
DESCRIPTION

1. TITLE OF THE INVENTION

NOVEL N-SUBSTITUTED-1-DEOXYNOJIRIMYCIN DERIVATIVE AND
CANCER CELL ANTIMETASTATIC AGENT INCLUDING THE SAME

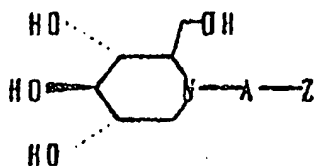
2. PATENT CLAIMS

1. An N-substituted-1-deoxynojirimycin derivative
represented by the following formula,



wherein A represents a hydrocarbon group of 3 to 5 carbon atoms optionally substituted with hydroxyl, alkyl halide or alkoxy group, the hydrocarbon group optionally comprising a double or triple bond, and Z represents phenyl, fluorinated phenyl, biphenyl, cycloalkyl or halogenated alkyl group.

2. A cancer cell antimetastatic agent characterized by an active ingredient which is an N-substituted-1-deoxynojirimycin derivative represented by the following formula or an addition salt thereof with a pharmaceutically acceptable acid,



wherein A represents a hydrocarbon group of 3 to 5 carbon atoms optionally substituted with hydroxyl, alkyl halide or alkoxy group, the hydrocarbon group optionally comprising a double or triple bond, and Z represents phenyl, fluorinated phenyl, biphenyl, cycloalkyl or halogenated alkyl group.

3. DETAILED DESCRIPTION OF THE INVENTION

[Industrial Field of Application]

The present invention relates to a novel N-substituted-1-deoxynojirimycin derivative which inhibits formation of cancer cell metastases and a cancer cell antimetastatic agent containing the same as the active ingredient.

[Conventional Technique]

Various anticancer agents are currently in use. Majority of them are drugs which kill cancer cells or let human immune system destroy them, but a drug effective for fundamental treatment of cancers has not been obtained yet.

Solid cancers, to which chemotherapeutic agents have low effectiveness, are treated with physical therapies

such as surgery or radiotherapy, and the success rate is greatly improved from a viewpoint of removing primary cancer. It is however also true that metastases of cancer cells are induced on the other side.

[Problem to be Solved by the Invention]

As described above, metastasis of cancer cells are the biggest problem in conventional cancer treatments which affects prognosis of patients with cancer.

Therefore, it is currently desired the most to develop an anticancer agent which can enhance suppression of cancer cell metastasis.

In order to achieve the above object, it is the purpose of the present invention to provide a substance which effectively suppresses cancer cell metastases and a cancer cell antimetastatic agent containing the same as the active ingredient.

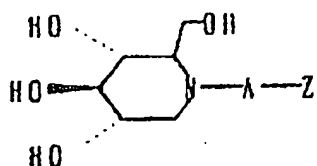
[Means for Solving the Problem]

The present inventors found N-substituted-1-deoxynojirimycin derivatives having a cancer cell antimetastatic effect prior to the present invention, and disclosed them in Japanese patent application publication Nos. Sho63-31095, Sho63-93673, Sho63-97454, Sho63-104850, Sho63-147815 and Sho63-147816.

The present inventors further synthesized novel N-

substituted derivatives of 1-deoxynojirimycin and broadly evaluated them, and then found a group of novel compounds having a strong cancer cell antimetastatic effect. The present invention has been thus accomplished.

The present invention is an N-substituted-1-deoxynojirimycin derivative represented by formula 1, and a cancer cell antimetastatic agent containing the compound or the addition salt thereof with a pharmaceutically acceptable acid as the active ingredient,



(1)

wherein A represents a hydrocarbon group of 3 to 5 carbon atoms optionally substituted with hydroxyl, alkyl halide or alkoxy group, the hydrocarbon group optionally comprising a double or triple bond, and Z represents phenyl, fluorinated phenyl, biphenyl, cycloalkyl or halogenated alkyl group.

The N-substituted-1-deoxynojirimycin derivative shown by formula 1 of the present invention is a novel substance which has not ever described in documents.

The following substances are examples of the compounds included in the novel N-substituted-1-deoxynojirimycin derivative:

N-(3-methoxymethyl-3-phenyl-2-propeny)-1-

deoxynojirimycin,

N-(3-phenyl-3-trifluoromethyl-2-propenyl)-1-

deoxynojirimycin,

N-[3-(4-fluorophenyl)-2-propenyl]-1-deoxynojirimycin,

N-[3[(3-fluorophenyl)-2-propenyl]-1-deoxynojirimycin,

N-[3[(2-fluorophenyl)-2-propenyl]-1-deoxynojirimycin,

N-[3-(4-biphenylpropyl)]-1-deoxynojirimycin,

N-[3-(4-fluorophenyl)-propyl]-1-deoxynojirimycin,

N-(3-cyclohexylpropyl)-1-deoxynojirimycin,

N-(3-phenyl-2-propenyl)-1-deoxynojirimycin,

N-(2,3-dihydroxy-3-phenylpropenyl)-1-deoxynojirimycin,

N-(6,6,6-trifluorohexyl)-1-deoxynojirimycin,

N-(5,5,5-trifluoropentyl)-1-deoxynojirimycin, and

N-(4,4,4-trifluorobutyl)-1-deoxynojirimycin.

When the N-substituted-1-deoxynojirimycin derivative of the present invention is used as a cancer cell antimetastatic agent, the pharmaceutically acceptable acid addition salt thereof includes addition salts of:

inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid and phosphoric acid;

organic acids such as formic acid, acetic acid, propionic acid, succinic acid, glycolic acid, lactic acid, malic acid, tartaric acid, citric acid, maleic acid, fumaric acid, benzoic acid, salicylic acid and methanesulfonic acid; and also amino acids such as asparaginic acid and glutamic acid.

All compounds of the present invention are novel compounds which have not ever described in documents. According to the most general synthesis method thereof, 1-deoxynojirimycin (see Tetrahedron, 24, 2125(1968)) is used as the raw material, which is obtained by reducing nojirimycin-(5-amino-5-deoxy-D-glucopyranose) (see Japanese patent application publication No. Sho43-760) which is a metabolite of an actinomycete found by the present inventors. Specifically, the N-substituted A-Z group of formula 1 of the present invention may be introduced by heating or leaving at room temperature 1-deoxynojirimycin with an aralkyl- or aralkenylation agent typrified by aralkyl halide or alkenyl halide, aralkylsulfonate ester or aralkenylsulfonate ester, etc. in polar solvent such as alcohols, dimethylformamide, dimethylacetoamide, dimethylsulfoxide, sulfolane and the mixture thereof in the presence of a deoxidizing agent such as alkali hydroxide, alkali carbonate, alkali bicarbonate, suitable organic amines, etc. It is also possible to employ a method such that the raw material is 1-deoxynojirimycin whose hydroxyl group is protected by a suitable protecting group, for example acetyl, benzoyl, tetrahydropyranyl, t-butyldimetylsilyl, or the like, and is subjected to the N-substitution reaction followed by deprotection. Furthermore, also available are: a method to carry out so-called reductive alkylation by use of an

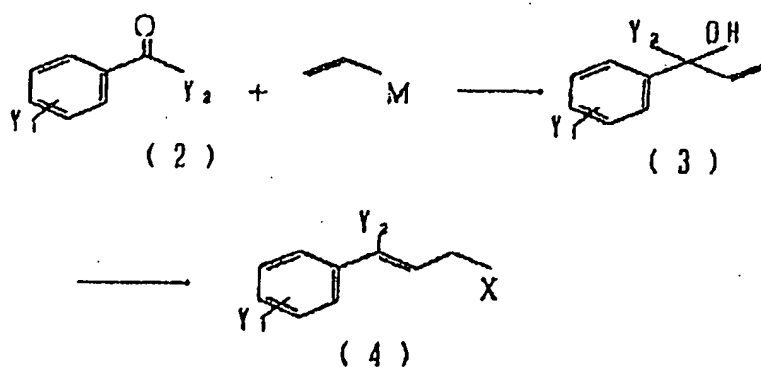
agent with carbonyl group as an reactive agent in hydrogen atmosphere under a reductive condition, for example conditions in the presence of formic acid, sodium cyanoborohydride, sodium borohydride or a suitable metal catalyst of platinum oxide, palladium or Raney nickel; and a method to obtain an objective product by reducing an amide compound of 1-deoxynojirimycin with aralkylcarbonic acid or aralkenylcarbonic acid. According to need, these compounds are subjected to a general purification procedure such as recrystallization, column chromatography, etc., so as to obtain the compound of formula 1 of the present invention.

The substitution group of the compound of the present invention may be formed and introduced by any method suitable for the purpose. The following five production methods are given as suitable methods to produce an aralkyl-, aralkenyl- or aralkynylation agent for constructing the A-Z group of formula 1.

[Production Method 1]

Compound 3 may be synthesized by the reaction of compound 2 with a vinyl-metal compound, for example vinylmagnesium chloride, divinylmagnesium bromide, vinylmagnesium iodide, vinyl lithium, divinylzinc, divinylcopper, divinylcesium, or the like, in nonpolar solvent, preferably in ether, tetrahydrofuran or dioxane, at -50°C to room temperature for 10 minutes to 24 hours.

Compound 4 may be synthesized by the reaction of compound 3 with hydrochloric acid, hydrobromic acid, oxalyl chloride, thionyl halide, oxyphosphorus halide, phosphorus trihalide, phosphorus pentahalide, tri-substituted phosphine-carbon tetrahalide, allyl- or alkylsulfonyl halide without solvent or in solvent such as benzene, toluene, ether, methylene chloride, acetonitrile, etc. at 0°C to 100°C for 30 minutes to 24 hours, the reaction being accompanied with transfer of the allyl alcohol part of compound 3.



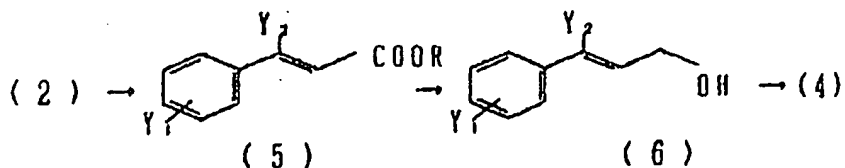
In the formula, Y_1 represents hydrogen atom, halogen atom, aralkyl or hydroxyl group, Y_2 represents hydrogen atom, halogen atom, aralkyl, alkoxy or halogen-substituted alkyl group, X represents halogen atom or alkyl- or allylsulfonyloxy group. The halogen atom denotes chlorine, brome, iodine atom, etc., and the alkyl- or allylsulfonyloxy group denotes methane sulfonyloxy, trifluoromethane sulfonyloxy, p-toluene sulfonyloxy group, etc. M represents mono- or divalent metal or the salt

thereof, and the metal denotes lithium, sodium, potassium, magnesium, zinc, cesium or copper.

[Production Method 2]

Unsaturated ester 5 is synthesized by the reaction of compound 2 with carboalkoxymethylene tri-substituted phosphorane in suitable solvent, preferably benzene, toluene, ether, tetrahydrofuran, dioxane, methylene chloride, chloroform, methanol and ethanol, at 0°C to 60°C for 10 minutes to 24 hours, or with diaralkylphosphonoacetic acid aralkylester in the presence of a suitable base, for example sodium hydride, potassium hydride, alkali hydride or alkali carbonate, at 0°C to 60°C for 10 minutes to 24 hours. Compound 6 may be synthesized by the reaction of compound 5 with a suitable metal hydride complex reductant, preferably lithium aluminum hydride, diisobutylaluminum hydride, sodium bis(2-methoxyethoxy)aluminum hydride, or the like, in suitable aprotic solvent, preferably ether, tetrahydrofuran or dioxane, at -78°C to -100°C for 30 minutes to 18 hours. Compound 4 may be synthesized by the reaction of compound 6 with hydrochloric acid, hydrobromic acid, oxalyl chloride, thionyl halide, phosphorus trihalide, phosphorus pentahalide, tri-substituted phosphine-carbon tetrahalide, allyl- or alkylsulfonyl halide without solvent or in solvent such as benzene, toluene, ether, methylene chloride, acetonitrile etc. at 0°C to 100°C for 30 minutes

to 24 hours.

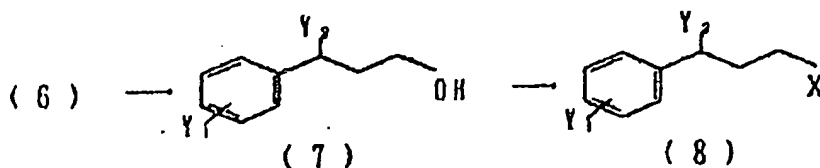


In the formula, Y_1 and Y_2 represent the same as above, and R represents a protection group of carboxyl such as alkyl.

[Production Method 3]

Saturated alcohol 7 may be synthesized by the reduction of alkenylalcohol 6 obtained in production method 2 in the presence of a metal catalyst, for example palladium-carbon, platinum, Raney nickel, or the like, in suitable organic solvent, for example methanol, ethanol, acetic acid, tetrahydrofuran, ethyl acetate, or the like, in hydrogen atmosphere for 30 minutes to 24 hours.

Compound 8 may be synthesized by the reaction of compound 7 in solvent such as hydrobromic acid, oxalyl chloride, thionyl halide, phosphorous oxyhalide, phosphorous trihalide, phosphorous pentahalide, tri-substituted phosphine-carbon tetrahalide, allyl- or alkylsulfonyl halide, etc. at 0°C to 100°C for 30 minutes to 24 hours.

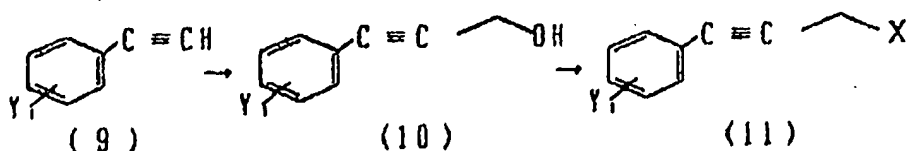


In the formula, Y_1 , Y_2 and X represent the same as

above.

[Production Method 4]

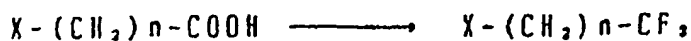
Alkynylalcohol 10 may be synthesized by acetylidation of 1-allylacetylene derivative 9 with a suitable base, for example n-butyllithium, lithium diisopropylamide, sodium amide or the like, followed by reaction with formalin. Compound 11 may be synthesized by the reaction of compound 10 with oxalyl chloride, thionyl halide, phosphorous oxyhalide, phosphorous trihalide, phosphorous pentahalide, tri-substituted phosphine-carbon tetrahalide or allyl- or alkylsulfonyl halide without solvent or in solvent such as benzene, toluene, ether, methylene chloride, acetonitrile, etc. at 0°C to 100°C for 30 minutes to 24 hours.



In the formula, Y1, Y2 and X represent the same as above.

[Production Method 5]

As a production method of a terminally halogenated alkylation agent, for example, a trifluoromethyl derivative 13 may be synthesized by treating ω-halogenated fatty acid 12 with a suitable fluorinating agent, for example sulfur tetrafluoride (Angew. Chem. Internat. Ed., 1, 467(1962)).



(12)

(13)

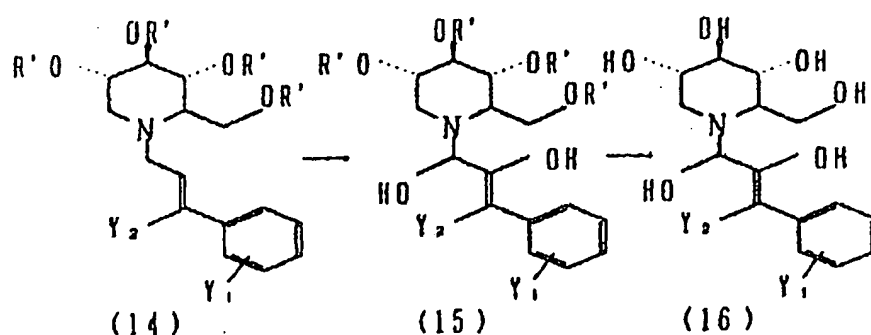
In the formula, X represents the same as above.

The N-substituted A-Z group of the compound of formula 1 in the present invention may be introduced by heating or leaving at room temperature with an aralkyl- or aralkenylation agent typified by the aralkyl halide or aralkenyl halide produced by the above production methods 1 to 5 and aralkylsulfonate ester or aralkenylsulfonate ester in polar solvent such as alcohols, dimethylformamide, dimethylacetamide, dimethylsulfoxide, sulfolane, etc. or the mixture thereof in the presence of a deoxidizing agent such as alkali hydroxide, alkali carbonate, alkali bicarbonate or suitable organic amines. It is also possible to employ a method such that the raw material is 1-deoxynojirimycin whose hydroxyl is protected by a suitable protecting group, for example acetyl, benzoyl, tetrahydropyranyl, t-butyldimethylsilyl, or the like, and N-substitution reaction is carried out followed by deprotection. Among the compounds included in the present invention, the ones of formula 1 where A is a hydroxyl-substituted hydrocarbon may be produced according to the following production method 6.

[Production method 6]

Objective product 16 may be obtained by the reaction

of N-substituted-1-deoxynojirimycin derivative 14, which may be synthesized by the reaction of the alkenylation agent synthesized according to production method 1 or 2 with 1-deoxynojirimycin or 1-deoxynojirimycin with protected hydroxyl, with a suitable oxidization agent, for example osmium tetroxide, or the like.



In the formula, Y_1 and Y_2 represent the same as above, R' represents hydrogen atom, acetyl, benzil, benzoyl, pivaloyl, t-butyldimethylsilyl or tetrahydropyranyl group.

Next, production examples of the N-substituted-1-deoxynojirimycin derivative of the present invention are shown.

[Production Example 1]:

N-(3-phenyl-3-trifluoromethyl-2-propenyl)-1-deoxynojirimycin

[Step 1]:

3-phenyl-3-trifluoromethyl-2-propene-1-ol

A solution of 1.74 g (10.0 mmol) 2,2,2-trifluoroacetophenone, which was dissolved in 10 ml of

tetrahydrofuran, was cooled to -78°C , and 1M vinylmagnesiumbromide solution in tetrahydrofuran was added dropwise. Following to the addition, the solution was stirred for 3 hours, and further for 1 hour without the cool bath. Water was added to decompose excess reagent in ice bath, and the solvent was then distilled away. 10 ml of 2N sulfuric acid was added to the residue, and extraction was carried out with ethyl acetate. The extract was washed with water, dried and then concentrated. The residue was purified with silica gel column chromatography (eluting solvent: ether-hexane (1:10)), so as to obtain 1.66 g (82%) of oily product.

NMR (CDCl_3) δ

2.61 (s, 1H), 5.52 (d, 1H), 5.62 (d, 1H),
6.43 (dd, 1H), 7.25-7.70 (m, 5H)

[Step 2]:

1-bromo-3-phenyl-3-trifluoromethyl-2-propene

606 mg (3.00 mmol) of 3-phenyl-3-trifluoromethyl-2-propene-1-ol and 943 mg (3.60 mmol) of triphenylphosphine were dissolved in 4 ml of acetonitrile and cooled in ice bath. 1.26 g (3.80 mmol) of carbon tetrabromide was then added in several parts. The solution was stirred for 1 hour in ice bath, and then further stirred overnight at room temperature. The reaction was diluted with 10 ml of ether, deposited solid was filtered off, and the filtrate was concentrated. The obtained residue was purified with

silica gel column chromatography (eluting solvent: hexane), so as to obtain 440 mg (55%) of oily product.

NMR (CDCl₃) δ

3.80 (dq, 2H), 8.62 (tq, 1H), 7.20-7.60 (m, 5H)

[Step 3]:

N-(3-phenyl-3-trifluoromethyl-2-propenyl)-1-deoxynojirimycin

163 mg (1.00 mmol) of deoxynojirimycin and 318 mg (1.20 mmol) of 1-bromo-3-phenyl-3-trifluoromethyl-2-propene were dissolved in 5 ml of dimethylformamide. 207 mg (1.50 mmol) of potassium carbonate was added and the solution was stirred for 8 hours at room temperature. Saturated salt solution was added to the reaction mixture, and extraction was carried out with n-butanol. The extract was concentrated under reduced pressure, and the residue was purified with silica gel column chromatography (eluting solvent: chloroform-methanol (10:1)), so as to obtain 311 mg (90%) of colorless solid product.

NMR (CD₃OD) δ

2.15 (m, 2H), 3.10 (dd, 1H), 3.16 (t, 1H),

3.31 (m, 1H), 3.42 (t, 1H), 3.53 (m, 1H),

3.78 (dd, 1H), 3.96 (ABX type, 2H),

6.72 (t, 1H), 7.32 (m, 2H), 7.46 (m, 3H)

[Production Example 2]:

N-(3-metoxymethyl-3-phenyl-2-propenyl)-1-deoxynojirimycin

The synthesis was carried out by use of 1-bromo-3-

metoxymethyl-3-phenyl-2-propene which was synthesized in the same manner as production method 1.

NMR (CD₃OD) δ

2.13 (m, 2H), 3.06 (dd, 1H), 3.16 (t, 1H),

3.34 (m, 1H), 3.44 (t, 1H), 3.31 (m, 1H),

3.38 (s, 3H), 3.76 (dd, 1H),

3.97 (ABX type, 2H), 4.16 (s, 2H),

6.06 (t, 1H), 7.15-7.50 (m, 5H)

[Production example 3]:

N-[3-(4-fluorophenyl)-2-propenyl]-1-deoxynojirimycin

[Step 1]:

Methyl-3-(4-fluorophenyl)-2-propenoate

1.24 g (10.0 mmol) of 4-fluorobenzaldehyde was dissolved in 20 ml of methylene chloride. 3.67 g (11.0 mmol) of carbomethoxymethylenetriphenylphosphorane was added, and the mixture was stirred for 3 hours at room temperature. Solid was filtered off, the filtrate was concentrated, and the residue was purified with silica gel chromatography (eluting solvent: ethyl acetate-hexane (1:4)), so as to obtain 1.61 g (90%) of colorless needle crystal.

NMR (CDCl₃) δ

4.30 (d, 2H), 6.25 (m, 1H), 6.55 (d, 1H),

6.95 (m, 2H), 7.35 (m, 2H)

[Step 2]:

3-(4-fluorophenyl)-2-propene-1-ol)

1.61 g (9.00 mmol) of methyl-3-(4-fluorophenyl)-2-propenoate was dissolved to 50 ml of ether, and the solution was dropwise added to 205 mg (5.40 mmol) of lithium aluminum hydride suspended in 3 ml of ether in ice bath. Stirring for 30 min at room temperature after the addition, excess reagent was then decomposed with water, and solid was filtered off. The filtrate was concentrated, so as to obtain 1.33 g (97%) of 3-(4-fluorophenyl)-2-propene-1-ol.

NMR (CDCl₃) δ

4.52 (d, 2H), 6.31 (m, 1H), 7.01 (m, 2H),
7.45 (m, 2H)

[Step 3]:

1-bromo-3-(4-fluorophenyl)-2-propene

1.34 g (8.82 mmol) of 3-(4-fluorophenyl)-2-propene-1-ol and 4.26 g (11.5 mmol) of tri-n-octylphosphine was dissolved in 20 ml of ether, and 3.52 g (10.6 mmol) of carbon tetrabromide was added in several parts in ice bath. After stirring for 30 min at room temperature, precipitate was filtered off, the filtrate was concentrated, and the residue was purified with silica gel column chromatography (eluting solvent: hexane), so as to obtain 1.61 g (85%) of colorless oily product.

NMR (CDCl₃) δ

3.35 (d, 2H), 6.30 (m, 1H), 7.00 (m, 2H),
7.40 (m, 2H)

Mass m/z 214, 216

[Step 4]:

N-[3-(4-fluorophenyl)-2-propenyl]-1-deoxynojirimycin

1.61 g (7.5 mmol) of 1-bromo-3-(4-fluorophenyl)-2-propene and 1.22 g (7.5 mmol) of 1-deoxynojirimycin were dissolved in 10 ml of dimethylformamide. 3.12 g (22.5 mmol) of Potassium carbonate was added and stirred 24 hours at room temperature. Water was added to the reaction mixture, and extraction was carried out with n-butanol. After distilling away the solvent, the residue was purified with silica gel column chromatography (eluting solvent: chloroform-methanol (10:1)), so as to obtain 1.36 g (61%) of pale yellow solid product.

NMR (CD₃OD) δ

2.4-4.2 (m, 16H), 6.40 (m, 1H), 6.7 (m, 1H),
7.10 (m, 2H), 7.55 (m, 2H)

Mass m/z 298 (FD, M+1)

[Production Example 4]:

N-[3-(3-fluorophenyl)-2-propenyl]-1-deoxynojirimycin

The synthesis was carried out in the same manner as production example 3.

NMR (CD₃OD) δ

2.15 (m, 2H), 3.04 (dd, 1H), 3.14 (t, 1H),
3.2-3.35 (m, 1H), 3.39 (t, 1H),
3.49 (m, 1H), 3.68 (dd, 1H),
3.94 (ABX type, 2H), 6.41 (dt, 1H),

6.59 (d, 1H), 6.95 (dt, 1H), 7.16 (dd, 1H),

7.21 (d, 1H), 7.31 (ddd, 1H)

Mass m/z 298 (FD, M+1)

[Production Example 5]:

N-[3-(2-fluorophenyl)-2-propenyl]-1-deoxynojirimycin

The synthesis was carried out in the same manner as production example 3.

NMR (CD₃OD) δ

2.1-2.25 (m, 2H), 3.06 (dd, 1H),

3.14 (t, 1H), 3.24-3.35 (m, 1H),

3.39 (t, 1H), 3.50 (m, 1H), 3.71 (m, 1H),

3.94 (ABX type, 2H), 6.45 (dt, 1H),

6.72 (d, 1H), 7.0-7.16 (m, 2H),

7.2-7.28 (m, 1H), 7.53 (dt, 1H)

Mass m/z (FD, M+1)

[Production Example 6]:

N-[3-(4-biphenyl)propyl]-1-deoxynojirimycin

[Step 1]:

1.10 g (6.00 mmol) of methyl-3-(4-biphenyl)acrylate-4-biphenylcarboxyaldehyde was dissolved in 20 ml of dichloroethane. 3.03 g (9.10 mmol) of carbomethoxymethylenetriphenylphosphorane was added, and the solution was stirred for 1 hour at room temperature. After distilling away the solvent, the residue was purified with silica gel column chromatography (eluting solvent: ether-hexane (1:10)), so as to obtain 1.12 g

(78%) of colorless crystal.

NMR (CDCl₃) δ

3.83 (s, 3H), 6.49 (d, 1H), 7.30-7.60 (m, 9H),

7.75 (d, 1H)

[Step 2]:

Methyl-3-(4-biphenyl)propionate

1.40 g (4.40 mmol) of methyl-3-(4-biphenyl)acrylate was dissolved in 50 ml of ethyl acetate. 70 mg of 10% Pd-C was added to carry out catalytic reduction under ambient pressure for 12 hours. After filtering off the catalyst, the solvent was distilled away so as to obtain 1.01 g (97%) of colorless oily product.

NMR (CDCl₃) δ

2.68 (t, 2H), 3.00 (t, 2H), 3.68 (s, 3H),

7.20-7.70 (m, 9H)

[Step 3]:

3'-(4-biphenyl)-1-propanol

To suspension of 110 mg (2.90 mmol) lithium aluminum hydride in 10 ml of ether, solution of 1.01 g (4.20 mmol) of methyl-3-(4-biphenyl)propionate in 35 ml of ether was added dropwise in ice bath. After stirring for 1 hour at the same temperature, excess reagent was decomposed with water, inorganic product was filtered off, and the filtrate was dried and concentrated, so as to obtain 861 mg (96%) of colorless crystal.

NMR (CDCl₃) δ

1.56 (br, 1H), 1.94 (m, 2H), 2.77 (m, 2H),
3.71 (m, 2H), 7.15-7.76 (m, 9H)

[Step 4]:

3-(4-biphenyl)-1-bromopropane

419 mg (2.00 mmol) of 3-(4-biphenyl)-1-propanol and 629 mg (2.40 mmol) of triphenylphosphine was dissolved in 10 ml of ether. 930 mg (2.80 mmol) of carbon tetrabromide was added in ice bath in several parts. After stirring for 1 hour at room temperature, precipitate was filtered off, the filtrate was concentrated, and the residue was purified with silica gel column chromatography (eluting solvent: hexane), so as to obtain 506 mg (92%) of colorless oily product.

NMR (CDCl₃) δ

2.20 (quin, 2H), 2.83 (t, 2H), 3.44 (t, 2H),
7.23-7.65 (m, 9H)

[Step 5]:

N-[3-(4-biphenyl)propyl]-1-deoxynojirimycin

140 mg (0.50 mmol) of 3-(4-biphenyl)-1-bromopropane and 82 mmol (0.5 mmol) of 1-deoxynojirimycin were dissolved in 1 ml of dimethylformamide. 136 mg (1.00 mmol) of potassium carbonate was added and heated at 80°C for 4 hours. Water was added, and the reaction mixture was acidified with hydrogen chloride and washed with ether. The aqueous phase was alkalized with ammonia, and extraction was carried out with n-butanol. After removing

the solvent, the residue was purified with silica gel column chromatography (eluting solvent: chloroform-methanol (10:1), so as to obtain 117 mg (66%) of solid product.

NMR (CD₃OD) δ

1.86 (m, 2H), 2.20 (br, 2H), 2.65 (m, 3H),
2.89 (m, 1H), 3.00 (m, 1H), 3.14 (t, 1H),
3.47 (m, 1H), 3.84 (d, 2H), 7.15-7.65 (m, 9H)

[Production Example 7]:

N-[3-(4-fluorophenylpropyl)]-1-deoxynojirimycin

The synthesis was carried out in the same manner as production example 6.

NMR (CD₃OD) δ

1.38 (m, 2H), 2.05-2.22 (m, 2H), 2.64 (m, 2H)
2.98 (dd, 1H), 3.13 (t, 1H), 3.30 (m, 1H),
3.38 (t, 1H), 3.45 (m, 1H),
3.64 (m, 1H), 3.85 (m, 2H), 7.18-7.35 (m, 4H)

[Production Example 8]

N-(3-cyclohexylpropyl)-1-deoxynojirimycin

The synthesis was carried out with the same manner as production example 6.

NMR (CD₃OD) δ

0.75-1.08 (m, 2H), 1.08-1.45 (m, 7H),
1.45-2.00 (m, 6H), 2.70-3.83 (m, 8H),
4.00 (ABX type, 2H)

[Production Example 9]:

N-(phenyl-2-propynyl)-1-deoxynojirimycin

[Step 1]:

1-phenyl-3-bromopropin

660 mg (5.00 mmol) of 1-phenyl-2-propin-1-ol and 4.98 g (15.0 mmol) of carbon tetrabromide were dissolved in 30 ml of tetrahydrofuran. 2.62 g (10.0 mmol) of triphenylphosphine was added thereto in ice bath in several parts. After stirring for 10 hours at room temperature, solid was filtered off and the filtrate was concentrated. The residue was purified with silica gel column chromatography (eluting solvent: hexane), so as to 181 mg (65%) of colorless oily product.

NMR (CDCl₃) δ

1.20 (br, 1H), 2.27 (s, 1H), 7.15-7.40 (m, 5H)

[Step 2]:

N-(phenyl-2-propynyl)-1-deoxynojirimycin

163 mg (1.00 mmol) of 1-deoxynojirimycin and 215 mg (1.10 mmol) of 1-phenyl-3-bromopropyne were dissolved in 3 ml of dimethylformamide. 166 mg (1.20 mmol) of potassium carbonate was added thereto and stirred for 8 hours at room temperature. Water was added, and the reaction mixture was acidified with hydrogen chloride and washed with ether. The aqueous phase was alkalized with ammonia, and extraction was carried out with n-butanol. After distilling away the solvent, the residue was purified with silica gel column chromatography (eluting solvent:

chloroform-methanol (10:1)), so as to obtain 181 mg (65%) of solid product.

NMR (CD₃OD) δ

2.31 (d, 1H), 2.57 (t, 1H), 2.98 (dd, 1H),

3.19 (t, 1H), 3.50 (t, 1H), 3.61 (m, 1H),

3.82 (ABX type, 2H), 3.98 (dd, 2H)

[Production Example 10]:

N-[(2,3-dihydroxy)-3-phenylpropyl]-1-deoxynojirimycin

[Step 1]:

N-(3-phenyl-2-propenyl)-1-deoxynojirimycin tetraacetate

1.42 g (7.20 mmol) of cinnamylbromide and 978 mg (6.00 mmol) of 1-deoxynojirimycin were suspended in 10 ml of dimethylformamide. 996 mg (7.20 mmol) of Potassium carbonate was added and heated at 60 to 65°C for 4 hours. After cooled, the mixture was diluted with 3 ml of methylene chloride. 3.06 g (30.0 mmol) of acetic anhydride and 2.37 g (30.0 mmol) of pyridine were added and stirred for 16 hours at room temperature. The reaction was diluted with 150 ml of ethyl acetate, washed with saturated sodium hydrogen carbonate solution and subsequently with water. After dried, the solvent was then distilled away. The residue was purified with silica gel column chromatography (eluting solvent: hexane-ethyl acetate (3:1)), so as to obtain 2.12 g (81%) of crystal.

NMR (CDCl₃) δ

2.01 (s, 6H), 2.03 (s, 3H), 2.09 (s, 3H),

2.38 (dd, 1H), 2.70 (dt, 1H), 3.25 (dd, 1H),
3.38 (dd, 1H), 3.59 (ddd, 1H), 4.19 (dd, 1H),
4.32 (dd, 1H), 4.90-5.20 (m, 3H), 6.22 (dt, 1H),
6.56 (d, 1H), 7.15-7.50 (m, 5H)

[Step 2]:

N-[(2,3-dihydroxy)-3-phenylpropyl]-1-deoxynojirimycin
tetraacetate

305 mg (0.70 mmol) of N-(3-phenyl-2-propenyl)-1-deoxynojirimycin tetraacetate and 98 mg (0.84 mmol) of N-methylmorpholine-N-oxide were dissolved in 8 ml of 50% acetone. 2 mg of osmium tetroxide was added and stirred for 2 hours. After adding 250 mg of sodium nitrite and 3 ml of water and stirring for 1 hours, the solution was diluted with 30 ml of water and extraction was carried out with ethyl acetate. After washed with water and dried, the solvent was distilled away. The residue was purified with silica gel column chromatography (eluting solvent: hexane-ethyl acetate (1:1)), so as to obtain 222 mg (68%) of caramel product. This compound was a mixture (2:1) of two stereoisomers.

NMR (CDCl₃) δ

2.32 (dd), 2.57 (dd), 2.70 (ABX type), 2.85 (dd),
2.97 (m), 3.11 (s), 3.12 (dd), 3.16 (s), 3.22 (dd),
3.82 (br), 4.13 (ABX type), 4.20 (ABX type),
4.48 (t), 4.53 (t), 4.86-5.12 (m),
7.2-7.4 (m, 5H)

[Step 3]:

N-[(2,3-dihydroxy)-3-phenylpropyl]-1-deoxynojirimycin

196 mg (0.42 mmol) of N-[(2,3-dihydroxy)-3-phenylpropyl]-1-deoxynojirimycin tetraacetate was dissolved in 5 ml of methanol. 3 mg of potassium carbonate was added and stirred for 3 hours at room temperature. After distilling away the solvent, the residue was purified with silica gel column chromatography (eluting solvent: chloroform-methanol (3:1)), so as to obtain 128 mg (98%) of colorless caramel product. This compound was a mixture (2:1) of two stereoisomers.

NMR (CD₃OD) δ

2.05 (dd), 2.17 (dd), 2.23-2.35 (m), 2.54 (dd),
2.87 (dd), 2.98 (dd), 3.10 (t), 3.14 (t),
3.2-4.0 (m), 4.50 (d), 4.68 (d),
7.15-7.50 (m, 5H).

Next, shown are results of evaluating cancer cell antimetastatic effect of the N-substituted deoxynojirimycin derivatives of the present invention.

[Effect Test]

[Test Method]

From melanoma B16 strain, which is a mouse tumor cell, a B16 high metastatic strain was selected for use based on the Fidler's method (Method in Cancer Research, 15, 339-439, 1978). Antimetastatic effect was evaluated based on the method of Kijima-Suda and others (Proc.,

Natl., Acad., Sci., U.S.A., 83, 1752-1756, 1986; Cancer Research, 46, 858-862, 1986.). First, the B16 high metastatic strain was seeded on Dulbecco's ME medium (DME medium) containing fetal bovine serum. N-substituted-1-deoxynojirimycin represented by general formula 1 was added, and the cells were cultured for 2 to 4 days at 37°C in the presence of 5% CO₂. The grown cells were peeled from the culture vessel with trypsin-EDTA solution. These cells were suspended in Dulbecco's balanced salt solution without Ca⁺⁺ and Mg⁺⁺ at 1x10⁶ cells/1 ml based on living cells.

Mice were injected with 0.1 ml of this suspension via tale vine to transplant the cells. After grown for 14 days, the lungs were extirpated by laparotomy. The number of the surface and internal metastatic nodes of B16 high metastatic strain formed on the lungs was counted and compared with the control which was not treated with the agent.

[Test Example 1]: Cellular Cytotoxicity

The B16 high metastatic strain was cultured in DME medium containing 10% fetal bovine serum at 37°C in the presence of 5% CO₂. The cells were peeled from the culture vessel with trypsin-EDTA solution, and suspended at 1x10⁴ cells per 1 ml. 150 µl of the suspension were added to and mixed with each 50 µl of test drug and control drug solution. The cells were then cultured for 4

days, and the living/dead thereof was observed under an inverted microscope to decide cellular cytotoxicity. The result is shown in Table 1.

Table 1

Used cell	B16 high metastasis strain	
Added drug	Concentration	Viability
Non-added		+
Compound of Production Example 9	10 $\mu\text{g/ml}$	+
	30 $\mu\text{g/ml}$	+
	100 $\mu\text{g/ml}$	+
Compound of Production Example 10	10 $\mu\text{g/ml}$	+
	30 $\mu\text{g/ml}$	+
	10 $\mu\text{g/ml}$	+
Compound of Production Example 7	10 $\mu\text{g/ml}$	+
	30 $\mu\text{g/ml}$	+
	100 $\mu\text{g/ml}$	+
Adriamycin (control)	0.1 $\mu\text{g/ml}$	-

"+" represents "living" and "-" represents "dead".

According to the test result, the compounds of the present invention did not have cellular cytotoxicity to B16 high metastatic strain.

[Test Example 2]: Antimetastatic Effect

B16 high metastatic strain was seeded to DME medium containing 10% fetal bovine serum. Each test drug was added at 30 μg per 1 ml, and the cells were cultured for 3 days at 37°C in the presence of 5% CO₂. The cells were peeled from the culture vessel in the same way as test example 1. These cells were suspended in Dulbecco's

balanced salt solution without Ca^{++} and Mg^{++} at 1×10^6 cells/1 ml based on living cells. BDF₁ Mice (8 weeks old, male) were injected with 0.1 ml thereof via tail vein to transplant the cells. After grown for 14 days, the lungs were extirpated by laparotomy. The number of the surface and internal metastatic nodes of B16 high metastatic strain formed in the lungs was counted. The result is shown in Table 2.

Table 2

Added drug	The number of lung metastatic nodes (average \pm standard deviation)
Non-added	207 \pm 47
Compound of Production Example 9 (30 $\mu\text{g/ml}$)	96 \pm 29
Compound of Production Example 10 (30 $\mu\text{g/ml}$)	60 \pm 18
Compound of Production Example 7 (30 $\mu\text{g/ml}$)	18 \pm 7

According to the result, the treatment with the compounds of the present invention greatly reduced the number of metastatic nodes formed in the lung.

The cancer cell antimetastatic agent of the present invention is oral or parenteral formulate containing the above N-substitued-1-deoxynojirimycin derivative, and clinically administered via vein, artery, skin, subcutaneous, intracutaneous, rectum or muscle, or orally. It is expected that direct administration to a tumor brings intense effect. The dose, which depends on

administration route, dosage form, and age, weight and condition of a patient, is basically 100 to 3,000 mg per day and given one or several times.

As the parenteral formulate, there can be given sterile aqueous and non-aqueous liquid formulation and emulsion formulation. As the base of the non-aqueous liquid formulation and emulsion formulation, there can be given propylene glycol, polyethylene glycol, glycerin, olive oil, corn oil, ethyl oleate, etc.

As the oral formulate, there can be given capsule, tablet, granule, powder, etc.

To these formulates, starch, lactose, mannite, ethylcellulose, sodium carboxymethylcellulose or the like is blended as excipient, and magnesium stearate or calcium stearate is added as lubricant. As binder, gelatin, gum arabic, cellulose ester, polyvinylpyrrolidone or the like is used.

Next, a formulation example of the present invention is described.

[Example]

N-[3-(4-fluorophenyl)-2-propenyl]-1-deoxynojirimycin: 200
mg

lactose: 130 mg

potato starch: 70 mg

polyvinylpyrrolidone: 10 mg

magnesium stearate: 2.5 mg

Lactose and potato starch were mixed and wetted uniformly with 20% solution of polyvinylpyrrolidone in ethanol. The mixture was filtered with 1 mm mesh, dried at 45°C, and filtered with 1 mm mesh again. The obtained granule was mixed with magnesium stearate, and shaped to tablets.

[Advantage of the Invention]

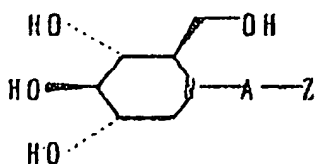
The present invention is a highly useful substance having cancer cell antimetastatic effect. The cancer cell antimetastatic agent containing this substance as the active ingredient solves the problem of cancer cell metastasis, which there is currently little countermeasure for and affects prognosis of patients with cancer the most, and is therefore a highly useful invention.

AMENDMENT

6. Content of Amendment

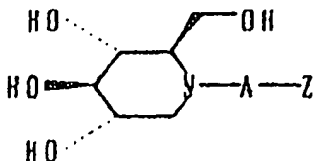
(1) The patent claims are amended as follows.

"1. An N-substituted-1-deoxynojirimycin derivative represented by the following formula,



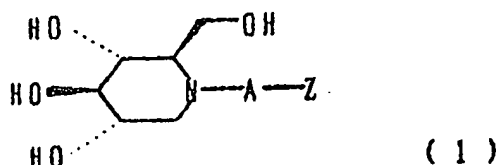
wherein A represents a hydrocarbon group of 3 to 5 carbon atoms optionally substituted with hydroxyl, alkyl halide or alkoxy group, the hydrocarbon group optionally comprising a double or triple bond, and Z represents phenyl, fluorinated phenyl, biphenyl, cycloalkyl or halogenated alkyl group.

2. A cancer cell antimetastatic agent characterized by an active ingredient which is an N-substituted-1-deoxynojirimycin derivative represented by the following formula or an addition salt thereof with a pharmaceutically acceptable acid,



wherein A represents a hydrocarbon group of 3 to 5 carbon atoms optionally substituted with hydroxyl, alkyl halide or alkoxy group, the hydrocarbon group optionally comprising a double or triple bond, and Z represents phenyl, fluorinated phenyl, biphenyl, cycloalkyl or halogenated alkyl group."

(2) On p.4 (p.4) of the description, formula 1 is amended as follows:



(3) On p.3, 1.12-14 (p.3, 1.10-12) of the description, "Therefore, it is ... cancer cell metastasis." is amended as follows.

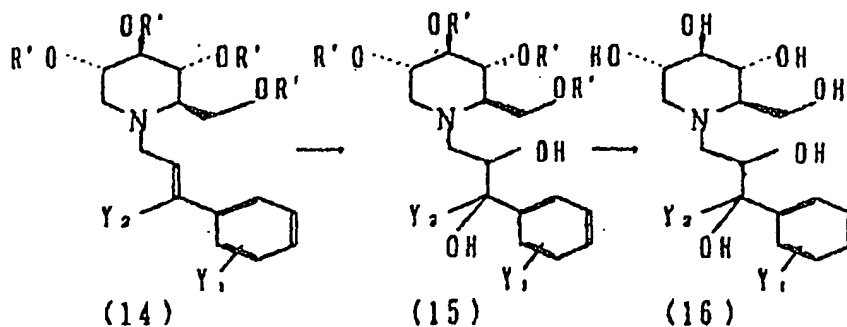
"Therefore, it is expected that suppression of cancer cell metastasis further improves the effectiveness of current cancer treatments."

(4) On p.15 in the 9th line from the bottom (p.12, 1.4-5) of the description, "... heating or leaving at room temperature with an aralkyl- or aralkenylation agent ..." is amended as follows.

"... heating or leaving at room temperature 1-

nojirimycin with an aralkyl- or aralkenylation agent ..."

(5) On p.16 (p.13) of the description, formulae (14), (15) and (16) are amended as follows.



NEW N-SUBSTITUTED-1-DEOXYNOJIRIMYCIN DERIVATIVE AND METASTASIS-INHIBITOR FOR CANCEROUS CELL

Publication number: JP2306962 (A)

Publication date: 1990-12-20

Inventor(s): KURIHARA HIROSHI; YOSHIDA SEISHI; TSURUOKA TSUTOMU; TSURUOKA TAKASHI; YAMAMOTO HARUO; FUKUYASU SHUNKAI

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Classification:

- International: C07D211/46; A61K31/445; A61P35/00; C07D211/00; A61K31/445; A61P35/00; (IPC1-7): A61K31/445; C07D211/46

- European:

Application number: JP19890127499 19890519

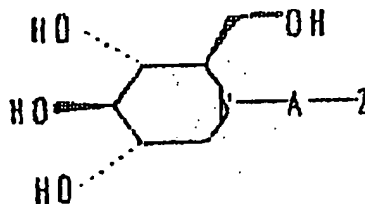
Priority number(s): JP19890127499 19890519

Abstract of JP 2306962 (A)

NEW MATERIAL: An N-substituted-1-deoxynojirimycin derivative expressed by the formula (A is 3-5C hydrocarbon may be substituted with OH, halogenated alkyl or alkoxy (said hydrocarbon may have double or triple bond); Z is phenyl, fluorine-substituted phenyl, biphenyl, cycloalkyl or halogen-substituted alkyl).

EXAMPLE: An N-(3-phenyl-3-trifluoromethyl-2-propenyl)-1-deoxynojirimycin. **USE:** Used as metastasis-inhibitor for cancerous cell.

PREPARATION: For instance, 1-deoxynojirimycin is reacted with various aralkylation agent or aralkenylation agent in the presence of deoxidizer such as alkali hydroxide to afford the compound expressed by the formula.



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⑩ 日本国特許庁(JP)

⑪ 特許出願公開

⑫ 公開特許公報(A) 平2-306962

⑬ Int. Cl.³

識別記号

庁内整理番号

⑭ 公開 平成2年(1990)12月20日

C 07 D 211/48
A 61 K 31/445

ADU

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審査請求 未請求 請求項の数 2 (全12頁)

⑯ 発明の名称 新規N-置換-1-デオキシノジリマイシン誘導体及びそれを含有する癌細胞転移抑制剤

⑰ 特 願 平1-127499

⑱ 出 願 平1(1989)5月19日

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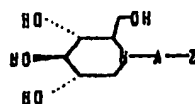
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明 細 書

1. 発明の名称 新規N-置換-1-デオキシノジリマイシン誘導体及びそれを含有する癌細胞転移抑制剤

2. 特許請求の範囲

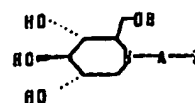
1. 式



式中、Aは水酸基、ハロゲン化アルキル基又はアルコキシ基で置換されてもよい炭素数3乃至5の炭化水素基を表し、この炭化水素基は二重又は三重結合を有していてもよい、Zはフェニル基、フッソ置換フェニル基、ビフェニル基、シクロアルキル基又はハロゲン置換アルキル基を表す。

で示されるN-置換-1-デオキシノジリマイシン誘導体。

2. 式



式中、Aは水酸基、ハロゲン化アルキル基、アルコキシ基で置換されてもよい炭素数3乃至5の炭化水素基を表し、この炭化水素基は二重又は三重結合を有していてもよい、Zはフェニル基、フッソ置換フェニル基、ビフェニル基、シクロアルキル基又はハロゲン置換アルキル基を表す。

で示されるN-置換-1-デオキシノジリマイシン誘導体又はその商理的に許容される酸との付加塩を有効成分とすることを特徴とする癌細胞転移抑制剤。

3. 発明の詳細な説明

(産業上の利用分野)

本発明は、癌細胞の転移形成を阻害する新規N-置換-1-デオキシノジリマイシン誘導体及びその物質を有効成分とする癌細胞転移抑制剤に関する。

(従来の技術)

現在使用されている制癌剤は種々あるが、その主体は、癌細胞を殺細胞させるか、人の免疫系を

介して死滅させる薬剤であり、癌の根本的な治療に対して有効な薬剤は未だ得られていない。

また、化学療法剤の有効性が低い癌形態に対しては外科手術、放射線療法等の物理的療法が行われ、原発癌の除去という点では成功率が大幅に向上している。しかし、転移癌細胞の転移を誘発することも事実である。

(発明が解決しようとする課題)

上述の如く、従来の癌治療において、癌細胞の転移が癌治療患者の予後を左右する最大の問題となっている。

従って、この癌細胞の転移を抑制することが高められる前癌病の発見は現在最も要望されている課題である。

本発明はこの課題を解決する癌細胞転移を有効に抑制する物質並びに同物質を有効成分とする癌細胞転移抑制剤を提供することを目的とするものである。

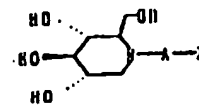
(課題を解決するための手段)

本発明者らは先に癌細胞転移抑制作用を有する

N-置換-1-デオキシノジリマイシン誘導体を見出し、特開昭63-31095号公報、特開昭63-93873号公報、特開昭63-97454号公報、特開昭63-104850号公報、特開昭63-147815号公報及び特開昭63-147816号公報に開示した。

本発明者らは更に1-デオキシノジリマイシンの新規なN-置換誘導体を合成し、その広範な評価を行ったところ、強い癌細胞転移抑制作用を有する一群の新規な化合物を見出し、本発明を完成した。

本発明は、式(1)



(1)

(式中、Aは水酸基、ハロゲン化アルキル基又はアルコキシ基で置換されてもよい炭素数3乃至5の炭化水素基を表し、この炭化水素基は二重又は三重結合を有してもよい、Zはフェニル基、フッソ置換フェニル基、ビフェニル基、シクロアルキル基又はハロゲン置換アルキル基を表す。)を示

されるN-置換-1-デオキシノジリマイシン誘導体、並びに同化合物又はその薬理的に許容される酸との付加塩を有効成分とする癌細胞転移抑制剤である。

本発明の式(1)で示されるN-置換-1-デオキシノジリマイシン誘導体は文献未載の新規物質である。

そして、このN-置換-1-デオキシノジリマイシン誘導体に含まれる化合物の例としては次のような物質が挙げられる。

N-(3-メトキシノチル-3-フェニル-2-プロペニル)-1-デオキシノジリマイシン

N-(3-フェニル-3-トリフロロメチル-2-プロペニル)-1-デオキシノジリマイシン

N-(3-(4-フロロフェニル)-2-プロペニル)-1-デオキシノジリマイシン

N-(3-(3-フロロフェニル)-2-プロペニル)-1-デオキシノジリマイシン

N-(3-(2-フロロフェニル)-2-プロペニル)-1-デオキシノジリマイシン

N-(3-(4-ビフェニルプロピル))-1-デオキシノジリマイシン

N-(3-(4-フロロフェニル)-プロピル)-1-デオキシノジリマイシン

N-(3-シクロヘキシルプロピル)-1-デオキシノジリマイシン

N-(3-フェニル-2-プロピニル)-1-デオキシノジリマイシン

N-(2,3-ジヒドロキシ-3-フェニルプロペニル)-1-デオキシノジリマイシン

N-(8,6,8-トリフロロヘキシル)-1-デオキシノジリマイシン

N-(5,5,5-トリフロロペンチル)-1-デオキシノジリマイシン

N-(4,4,4-トリフロロブチル)-1-デオキシノジリマイシン

また、本発明のN-置換-1-デオキシノジリマイシン誘導体を癌細胞転移抑制剤として使用する場合は薬理的に許容される酸の付加塩としては、塩酸、臭化水素酸、硫酸、硝酸、過硫酸等の無機酸、

酢酸、酢酸、プロピオン酸、コハク酸、グリコール酸、乳酸、リンゴ酸、酒石酸、クエン酸、マレイン酸、フマル酸、安息香酸、ナリチル酸、ノタンズルホン酸等の有機酸、更にはアスパラギン酸、グルタミン酸等のアミノ酸との付加塩が挙げられる。

本発明の化合物はいずれも文献未記載の新規化合物である。その合成法としては本発明者らによって見出された放電管の代用産物であるノリマイシン(5-アミノ-5-デオキシ-D-グルコピラノース)(特公昭43-760号公報参照)の還元により得られる1-デオキシノリマイシン

(Tetrahedron, 24, 2125(1968) 参照)を原料とする方法が最も一般的である。即ち、1-デオキシノリマイシンを各種のアルコール類、ジメチルホルムアミド、ジメチルアセトアミド、ジメチルスルホキシド、スルホラン等の極性溶媒又は、それらの混合溶媒中でアラルキルハライド、アルケニルハライド又はアラルキルスルホン酸エステル、アラルケニルスルホン酸エステル等で代表される

各級のアラルキル又はアラルケニル化試料と水酸化アルカリ、炭酸アルカリ、炭酸アルカリ又は適当な有機アミン類等の炭酸剤の存在下で室温又は加温することによって本発明の式(1)の化合物のN-置換A-Z基を導入することができる。また、水酸基を適当な保護基、例えばアセチル基、ベンゾイル基、チトラヒドロピラニル基、1-ブチルジメチルシリル基等で保護した1-デオキシノリマイシンを原料として用い、N-置換反応を行わたのち、脱保護する方法も採用される。また反応試薬としてカルボニル基を有する試薬を用いて還元的条件下、例えば銅酸、シアノ水素化ホウ素ナトリウム、水素化ホウ素ナトリウム或いは適当な金属触媒、例えば酸化白金、パラジウム、ランタニウム等の存在下、水素雰囲気下でいわゆる還元的アルキル化を行う方法、或いは1-デオキシノリマイシンとアラルキルカルボン酸、又はアラルケニルカルボン酸とのアミド還元して目的物を得る方法も使用することができる。これらの化合物は必要に応じて再結晶、カラムクロ

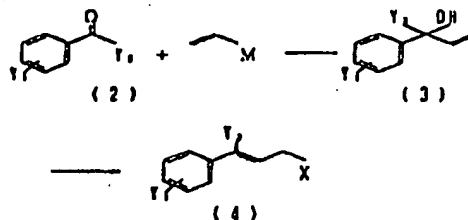
マトグラフィー等の一般的な精製法によって本発明の式(1)の化合物を得る。

本発明の化合物の置換基の形成及び導入に関しては合目的な適宜の方法によって合成することができる。式(1)のA-Z基を構築するためのアラルキル、アラルケニル、アラルケニル化剤の製造については適宜な方法として下記の5通りの製造法を示す。

製造法1

化合物(2)とビニル金属化合物、例えば塩化ビニルマグネシウム、臭化ビニルマグネシウム、炭化ビニルマグネシウム、ビニルリチウム、ジビニル亜鉛、ジビニル銅、ジビニルセシウム等とを無極性溶媒中、好ましくはエーテル、テトラヒドロフラン、ジオキサン中で-50℃〜室温、10分〜24時間反応させることによって化合物(3)を合成することができる。化合物(3)を塩酸、臭化水素酸、オキシリルクロリド、ハロゲン化チオニル、オキシハロゲン化銅、三ハロゲン化銅、五ハロゲン化銅、3置換ホスフィン-四ハロゲン化炭

素、アリル又はアルキルスルホニルハライドと無極性溶媒或いはベンゼン、トルエン、エーテル、塩化メチレン、アセトニトリル等の溶媒中で0℃〜100℃、30分〜24時間反応させることによって化合物(3)のアリルアルコール部分の転移を伴いながら化合物(4)を合成することができる。



(式中Yは水素原子、ハロゲン原子、アラルキル基、水酸基を表し、Yは水素原子、ハロゲン原子、アラルキル基、アルコキシ基、ハロゲン置換アルキル基を表す、Xはハロゲン原子、アルキル又はアリルスルホニロキシ基を表す。ハロゲン原子としては、塩素、臭素、炭素等を、アルキル又はアリルスルホニロキシ基としてはノタンズルホニルオキシ基、トリフロロノタンズルホニルオキシ基、

R-オトルエンスルホニルオキシ基等を示す。Mは1価又は2価の金属或いはその塩を表し、金属としてはリチウム、ナトリウム、カリウム、マグネシウム、亜鉛、セシウム、銅を示す)

製造法2

化合物(2)を適当な溶媒、好ましくはベンゼン、トルエン、エーテル、テトラヒドロフラン、ジオキサン、塩化メチレン、クロロホルム、メタノール、エタノール中カルボアルコキシメチレントリ置換ホスホランと0℃～60℃で10分～24時間反応させるか、又は適当な塩基、例えば水素化ナトリウム、水素化カリウム、水酸化アルカリ、炭酸アルカリの存在下、ジアルキルホスホノ酢酸アルキルエステルとを0℃～60℃で10分～24時間反応させ、不飽和エステル(5)を合成する。化合物(5)を適当な非プロトン性溶媒、好ましくはエーテル、テトラヒドロフラン、ジオキサン中、適当な水素化金属相還元剤、好ましくは水素化アルミニウムリチウム、ジイソブチルアルミニウムヒドリド、水素化ビス(2-メトキシエ

キシ)アルミニウムナトリウムと-78℃～100℃で30分～18時間反応させることによって化合物(6)を合成することができる。化合物(6)を塩酸、臭化水素酸、オキサリルクロリド、ハロゲン化チオニル、オキシハロゲン化剤、三ハロゲン化剤、五ハロゲン化剤、3置換ホスフィン-四ハロゲン化炭素、アリル又はアルキルスルホニルハライドと無溶媒或いはベンゼン、トルエン、エーテル、塩化メチレン、アセトニトリル等の溶媒中0℃～100℃で30分～24時間反応させることにより、化合物(4)を合成することができる。

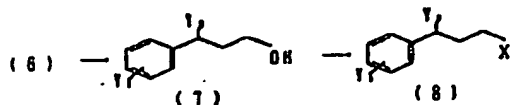


(式中、Y₁、Y₂は前記と同一意味を有し、Rはアルキル基などのカルボキシ基の保護基を表す)

製造法3

製造法2によって得られるアルケニルアルコール(6)を適当な有機溶媒、例えばメタノール、

エタノール、酢酸、テトラヒドロフラン、酢酸エステル等中で、金属触媒、例えばパラジウム-炭素、白金、ランタニウム等の存在下で水素雰囲気下で30分～24時間還元し、飽和アルコール(7)を合成することができる。化合物(7)を臭化水素酸、オキサリルクロリド、ハロゲン化チオニル、オキシハロゲン化剤、三ハロゲン化剤、五ハロゲン化剤、3置換ホスフィン-四ハロゲン化炭素、アリル又はアルキルスルホニルハライド等の溶媒中で0℃～100℃、30分～24時間反応させることにより、化合物(8)を合成することができる。

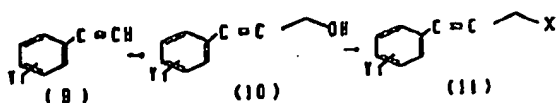


(式中、Y₁、Y₂、Xは前記と同一意味を有す)

製造法4

1-アリルアセチレン誘導体(9)を適当な塩基、例えばn-ブチルリチウム、リチウムジイソプロピルアミド、ナトリウムアミド等でアセチリ

ドとしたのち、ホルマリンと反応させることによって、アルケニルアルコール(10)を合成することができる。化合物(10)をオキサリルクロリド、ハロゲン化チオニル、オキシハロゲン化剤、三ハロゲン化剤、五ハロゲン化剤、3置換ホスフィン-四ハロゲン化炭素、アリル又はアルキルスルホニルハライドと無溶媒或いはベンゼン、トルエン、エーテル、塩化メチレン、アセトニトリル等の溶媒中0℃～100℃で30分～24時間反応させることにより、化合物(11)を合成することができる。

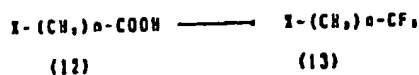


(式中Y₁、Y₂、Xは前記と同一意味を有す)

製造法5

定価ハロゲン置換アルキル化剤の製造法としては、例えばα-ハロゲン置換脂肪族(12)を適当なフッ素化剤、例えば四フッ化イオウ(Angew. Chem., International. Ed., 1, 467 (1962))で処理することに

よってトリフロロメチル誘導体(13)を合成することができる。



(式中、Xは前記と同一意義を有す)

以上の製造法1〜5で製造されたアラルキルハライド、アラルケニルハライド又はアラルキルスルホン酸エステル、アラルケニルスルホン酸エステル等で代表される各種のアラルキル又はアラルケニル化試料と各種アルコール類、ジメチルホルムアミド、ジメチルアセトアミド、ジメチルスルホキシド、スルホラン等の極性溶媒又はそれらの混合溶媒中、水酸化アルカリ、炭酸アルカリ、炭酸アルカリ又は、適当な有機アミン類等の脱酸剤の存在下で室温又は加温することによって、本発明の式(1)の化合物のN-置換A-Z基を導入することができる。また、水酸基を適当な保護基、例えばアセチル基、ベンゾイル基、テトラヒ

(式中、Y、Y'は前記と同一意義を有す、R'は水素原子、アセチル基、ベンジル基、ベンゾイル基、ヒバロイル基、1-ブチルジメチルシリル基、テトラヒドロピラニル基を示す)

次に本発明のN-置換-1-デオキシノジリマイシン誘導体の製造例を示す。

製造例1

N-(3-フェニル-3-トリフロロメチル-2-プロペニル)-1-デオキシノジリマイシン
工程1

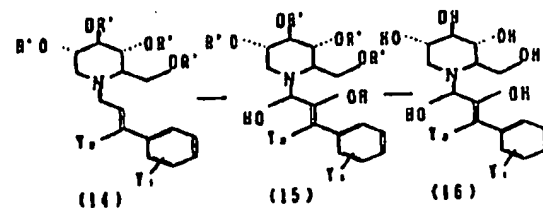
3-フェニル-3-トリフロロメチル-2-プロペン-1-オール

2, 2, 2-トリフロロアセトフェノン1.74g (10.0 mmol) をテトラヒドロフラン10mlに溶かした溶液を-78℃に冷却し、1Mピニルマグネシウムブライドテトラヒドロフラン溶液を加える。冷却終了後3時間同温度で攪拌後、冷却を取り去り1時間攪拌する。氷冷水を加えて過剰の試薬を分解した後、溶液を留去する。残液に2N硫酸10mlを加え、酢酸エチルで抽出する。抽出液を

ドロピラニル基、1-ブチルジメチルシリル基等で保護した1-デオキシノジリマイシンを原料として用い、N-置換反応を行わせた後、脱保護する方法も採用される。本発明に含まれる化合物のうち、式(1)中Aが水酸基で置換された炭化水素であるものについては、次に示す製造方法6に従って製造することができる。

製造法6

製造法1、或いは2に従って合成したアルケニル化剤と1-デオキシノジリマイシン或いは水酸基を保護した1-デオキシノジリマイシンとを反応させることによって合成することができるN-置換-1-デオキシノジリマイシン誘導体(14)を適当な酸化剤、例えば四酸化オスミウム等と反応させ目的物(16)を得ることができる。



水洗、乾燥後濃縮する。残液をシリカゲルカラムクロマトグラフィー(抽出溶媒:エーテル-ヘキサン(1:10))で精製し、1.66g(82%)の抽出物を得た。

$\text{NMR}(\text{CDCl}_3) \delta$

2.61(a, 1H), 5.52(d, 1H), 5.62(d, 1H), 6.43(dd, 1H), 7.25 ~ 7.70(a, 5H)

工程2

1-ブロモ-3-フェニル-3-トリフロロメチル-2-プロペン

3-フェニル-3-トリフロロメチル-2-プロペン-1-オール606 mg(3.00 mmol) とトリフェニルホスフィン943 mg(3.60 mmol) をアセトニトリル4mlに溶解し氷冷する。ここへ四氯化炭1.26g(3.80 mmol)を数回に分けて加える。氷冷下1時間攪拌した後、一夜室温下攪拌する。反応液をエーテル10mlで希釈し、析出する固体を濾過し、溶液を濃縮する。得られる残液をシリカゲルカラムクロマトグラフィー(抽出溶媒:ヘキサン)で精製し、440 mg(55%)の抽出物

を得た。

$^1\text{HNR}(\text{CDCl}_3)$ δ

3.80(dq, 2H), 8.62(tq, 1H), 1.20~1.60(m, 5H)

工程 3

N-(3-フェニル-3-トリフロロメチル-2-プロペニル)-1-デオキシノジリマイシン
デオキシノジリマイシン163 mg(1.00 mmol)と1-ブロモ-3-フェニル-3-トリフロロメチル-2-プロペン318 mg(1.20 mmol)をジメチルホルムアミド5 mlに溶解し、炭酸カリウム207 mg(1.50 mmol)を加えて室温下8時間攪拌する。反応混合物に飽和食塩水を加えてn-ブタノールで抽出する。抽出液を減圧下濃縮し、残渣をシリカゲルカラムクロマトグラフィー(抽出溶媒:クロロホルム-nブタノール(10:1))で精製し311 mg(90%)の無色固体を得た。

$^1\text{HNR}(\text{CD}_3\text{OD})$ δ

2.15(m, 2H), 3.10(dd, 1H), 3.16(t, 1H),
3.31(m, 1H), 3.42(t, 1H), 3.53(m, 1H),
3.78(dd, 1H), 3.98(ABX type, 2H).

メル)を塩化メチレン20 mlに溶解し、カルボノトキシメチレントリフェニルホスホラン3.67 g(11.0 mmol)を加え、室温下3時間攪拌した。固体を濾別し、母液を濃縮し、残渣をシリカゲルカラムクロマトグラフィー(抽出溶媒:酢酸エチル-ヘキサン(1:4))で精製し、無色針状品1.81 g(90%)を得た。

$^1\text{HNR}(\text{CDCl}_3)$ δ

4.30(d, 2H), 6.25(m, 1H), 6.55(d, 1H),
6.95(m, 2H), 7.35(m, 2H)

工程 2

3-(4-フロロフェニル)-2-プロペン-1-オール

ノチル-3-(4-フロロフェニル)-2-プロペノエート1.61 g(9.00 mmol)をエーテル50 mlに溶解し、氷冷下水酸化アルミニウムリチウム205 mg(5.40 mmol)をエーテル3 mlに懸濁したものに滴下する。滴下後室温下30分攪拌し、過剰の試薬を水で分解し、固体を濾別する。母液を濃縮し3-(4-フロロフェニル)-2-プロ

6.72(t, 1H), 7.32(m, 2H), 7.46(m, 3H)

製造例 2

N-(3-ノトキシメチル-3-フェニル-2-プロペニル)-1-デオキシノジリマイシン
製造例1と同様に合成した1-ブロモ-3-ノトキシメチル-3-フェニル-2-プロペンを用いて合成した。

$^1\text{HNR}(\text{CD}_3\text{OD})$ δ

2.13(m, 2H), 3.06(dd, 1H), 3.16(t, 1H),
3.34(m, 1H), 3.44(t, 1H), 3.51(m, 1H),
3.38(s, 3H), 3.76(dd, 1H),
3.97(ABX type, 2H), 4.16(s, 2H),
6.08(t, 1H), 7.15~7.50(m, 5H)

製造例 3

N-(3-(4-フロロフェニル)-2-プロペニル)-1-デオキシノジリマイシン

工程 1

ノチル-3-(4-フロロフェニル)-2-プロペノエート
4-フロロベンズアルデヒド1.24 g(10.0 mmol)

ベン-1-オール1.33 g(97%)を得た。

$^1\text{HNR}(\text{CDCl}_3)$ δ

4.52(d, 2H), 6.31(m, 1H), 7.01(m, 2H),
7.45(m, 2H)

工程 3

1-ブロモ-3-(4-フロロフェニル)-2-プロペン

3-(4-フロロフェニル)-2-プロペン-1-オール1.34 g(8.82 mmol)とトリ-n-オクタールホスフィン4.28 g(11.5 mmol)をエーテル20 mlに溶解し、氷冷下四臭化炭素3.52 g(10.6 mmol)を数回に分け加える。室温下30分攪拌した後、沈殿物を濾別し、母液を濃縮し残渣をシリカゲルカラムクロマトグラフィー(抽出溶媒:ヘキサン)で精製し1.61 g(85%)の無色油状物を得た。

$^1\text{HNR}(\text{CDCl}_3)$ δ

3.35(d, 2H), 6.30(m, 1H), 7.00(m, 2H),
7.40(m, 2H)

Mass m/z 214, 216

工程 4

N-(3-(4-フロロフェニル)-2-プロベニル)-1-デオキシノジリマイシン

1-プロモ-3-(4-フロロフェニル)-2-プロベニル 6.1 g (7.5 ミリモル) と 1-デオキシノジリマイシン 1.22 g (7.5 ミリモル) をジノチルホルムアミド 10 ml に溶解し、炭酸カリウム 3.12 g (22.5 ミリモル) を加え、室温下 24 時間攪拌した。反応混合物を水に注いで n-ブタノールで抽出する。抽出物を留去した後、残液をシリカゲルカラムクロマトグラフィー（抽出溶媒：クロロホルム-メタノール (10:1)）で精製し、1.36 g (61%) の淡黄色の固体を得た。

¹HMR(CD₃COO) δ

2.1 ~ 4.2 (m, 16H), 5.40 (m, 1H), 6.7 (m, 1H), 7.10 (m, 2H), 7.55 (m, 2H)

Mass m/z 298 (PD, M+1)

製造例 4

N-(3-(3-フロロフェニル)-2-プロベニル)-1-デオキシノジリマイシン

Mass m/z (PD, M+1)

製造例 6

N-(3-(4-ビフェニル)プロピル)-1-デオキシノジリマイシン

工程 1

ノチル-3-(4-ビフェニル)アクリレート 4-ビフェニルカルボキシアルデヒド 1.10 g (5.00 ミリモル) をジクロロエタン 20 ml に溶解し、カルボメトキシメチレントリフェニルホスホラン 3.03 g (9.10 ミリモル) を加え、室温下 1 時間攪拌する。抽出物を留去後、残液をシリカゲルカラムクロマトグラフィー（抽出溶媒：エーテル-ヘキサン (1:10)）で精製し、1.12 g (78%) の無色結晶を得た。

¹HMR(CDCl₃) δ

3.83 (s, 3H), 6.49 (d, 1H), 7.30 ~ 7.60 (m, 9H), 7.75 (d, 1H)

工程 2

ノチル-3-(4-ビフェニル)プロピオネート

製造例 3 と同様にして合成した。

¹HMR(CD₃COO) δ

2.15 (m, 2H), 3.04 (dd, 1H), 3.14 (t, 1H), 3.2 ~ 3.35 (m, 1H), 3.39 (t, 1H), 3.49 (m, 1H), 3.68 (dd, 1H), 3.94 (ABX type, 2H), 6.41 (dt, 1H), 6.59 (d, 1H), 6.95 (dt, 1H), 7.16 (dd, 1H), 7.21 (d, 1H), 7.31 (ddd, 1H)

Mass m/z 298 (PD, M+1)

製造例 5

N-(3-(2-フロロフェニル)-2-プロベニル)-1-デオキシノジリマイシン

製造例 3 と同様にして合成した。

¹HMR(CD₃COO) δ

2.1 ~ 2.25 (m, 2H), 3.06 (dd, 1H), 3.14 (t, 1H), 3.24 ~ 3.35 (m, 1H), 3.39 (t, 1H), 3.50 (m, 1H), 3.71 (m, 1H), 3.94 (ABX type, 2H), 6.45 (dt, 1H), 6.72 (d, 1H), 7.0 ~ 7.16 (m, 2H), 7.2 ~ 7.28 (m, 1H), 7.53 (dt, 1H)

ノチル-3-(4-ビフェニル)アクリレート 1.40 g (4.40 ミリモル) を酢酸エチル 50 ml に溶解し、10% Pd-C70 mg を加えて常圧下 12 時間加熱還元する。抽出物を留去後、抽出物を留去し、1.01 g (97%) の無色抽出物を得た。

¹HMR(CDCl₃) δ

2.68 (t, 2H), 3.00 (t, 2H), 3.68 (t, 3H), 7.20 ~ 7.70 (m, 9H)

工程 3

3'-(4-ビフェニル)-1-プロパロール 氷冷下、水酸化アルミニウムリチウム 110 mg (2.90 ミリモル) をエーテル 10 ml に懸濁した中へノチル-3-(4-ビフェニル)プロピオネート 1.01 g (4.20 ミリモル) をエーテル 35 ml に溶解したものを滴下する。同温度で 1 時間攪拌後、過剰の試薬を水で分解し、無機物を濾別、濾液を乾燥後、蒸留し、861 mg (96%) の無色結晶を得た。

¹HMR(CDCl₃) δ

1.56 (br, 1H), 1.94 (m, 2H), 2.77 (m, 2H), 3.71 (m, 2H), 7.15 ~ 7.76 (m, 9H)

工程4

3-(4-ビフェニル)-1-ブロモプロパン

3-(4-ビフェニル)-1-プロパノール

119 g (2.00 mmol) とトリフェニルホスフィン629 g (2.40 mmol) をエーテル10 ml に溶解し氷冷下四氯化炭素 930 g (2.80 mmol) を数回に分けて加える。室温下1時間攪拌した後、反応物を蒸留し、残渣を濃縮し残渣をシリカゲルカラムクロマトグラフィー(溶出溶媒:ヘキサン)で精製し506 g (92%) の無色油状物を得た。

¹H NMR(CDCl₃) δ

2.20 (quint, 2H), 2.83 (t, 2H), 3.44 (t, 2H), 1.23~1.65 (m, 9H)

工程5

N-(3-(4-ビフェニル)プロピル)-1-デオキシノジリマイシン

3-(4-ビフェニル)-1-ブロモプロパン140 g (0.50 mmol) と1-デオキシノジリマイシン82 g (0.5 mmol) をジメチルホルムアミド1 ml に溶解し、炭酸カリウム136 g (1.00 mmol) を加え、80℃、4時間加熱した。反応混合物を水に注いで塩酸性としエーテルにて洗浄、水相をアンモニアアルカリとし、n-ブタノールで抽出する。溶媒を除去した後、残渣をシリカゲルカラムクロマトグラフィー(溶出溶媒:クロロホルム-メタノール(10:1))で精製し117 g (86%) の固体を得た。

1.86 (m, 2H), 2.20 (br, 2H), 2.65 (m, 3H), 2.83 (m, 1H), 3.00 (m, 1H), 3.14 (t, 1H), 3.47 (m, 1H), 3.84 (d, 2H), 7.15~7.65 (m, 9H)

¹H NMR(CD₃COOD) δ

1.86 (m, 2H), 2.20 (br, 2H), 2.65 (m, 3H), 2.83 (m, 1H), 3.00 (m, 1H), 3.14 (t, 1H), 3.47 (m, 1H), 3.84 (d, 2H), 7.15~7.65 (m, 9H)

製造例7

N-(3-(4-フロロフェニル)プロピル)-1-デオキシノジリマイシン

製造例6と同様に合成した。

¹H NMR(CD₃COOD) δ

1.38 (m, 2H), 2.05~2.22 (m, 2H), 2.64 (m, 2H), 2.98 (dd, 1H), 3.13 (t, 1H), 3.30 (m, 1H), 3.38 (t, 1H), 3.45 (m, 1H), 3.64 (m, 1H), 3.85 (m, 2H), 7.18~7.35 (m, 4H)

製造例8

N-(3-シクロヘキシルプロピル)-1-デオキシノジリマイシン

製造例6と同様に合成した。

¹H NMR(CD₃COOD) δ

0.75~1.08 (m, 2H), 1.08~1.45 (m, 1H), 1.45~2.00 (m, 8H), 2.70~3.83 (m, 8H), 4.00 (ABX type, 2H)

製造例9

N-(フェニル-2-プロピニル)-1-デオキシノジリマイシン

工程1

1-フェニル-3-ブロモプロピン

1-フェニル-2-プロピン-1-オール660 g (5.00 mmol) と四氯化炭素4.98 g (15.0 mmol) をテトラヒドロフラン30 ml に溶解し、氷冷下トリフェニルホスフィン2.62 g (10.0 mmol) を数回に分けて加える。室温下10時間攪拌後、固体を蒸留し、残渣を濃縮する。残渣をシリカゲルカラムクロマトグラフィー(溶出溶媒:ヘキサ

ン)で精製し、181 g (65%) の無色油状物を得た。

¹H NMR(CDCl₃) δ

1.20 (br, 1H), 2.27 (s, 1H), 7.15~7.40 (m, 5H)

工程2

N-(フェニル-2-プロピニル)-1-デオキシノジリマイシン

1-デオキシノジリマイシン163 g (1.00 mmol) と1-フェニル-3-ブロモプロピン215 g (1.10 mmol) をジメチルホルムアミド3 ml に溶解し、炭酸カリウム166 g (1.20 mmol) を加え、室温下8時間攪拌する。反応混合物を水に注いで塩酸性としエーテルにて洗浄、水相をアンモニアアルカリとし、n-ブタノールで抽出する。溶媒を除去した後、残渣をシリカゲルカラムクロマトグラフィー(溶出溶媒:クロロホルム-メタノール(10:1))で精製し、181 g (65%) の固体を得た。

¹H NMR(CD₃COOD) δ

2.31 (d, 1H), 2.57 (t, 1H), 2.98 (dd, 1H),

3.19(t, 1H), 3.50(t, 1H), 3.61(o, 1H),
3.82(ABX type, 2H), 3.98(dd, 2H)

製造例10

N-[(2, 3-ジヒドロキシ)-3-フェニ
ルプロピル]-1-デオキシノジリマイシン

工程1

N-(3-フェニル-2-プロペニル)-1-
デオキシノジリマイシンテトラアセテート

シナミルプロピル 1.42 g (7.20ミリモル) と
1-デオキシノジリマイシン 978 mg (6.00ミリモ
ル) をジメチルホルムアミド 10 ml に溶解し、炭酸
カリウム 996 mg (7.20ミリモル) を加えて、4時
間、60~65℃に加熱する。冷却、塩化メチレン 3
ml で希釈し、無水酢酸 3.06 g (30.0ミリモル) と
ピリジン 2.37 g (30.0ミリモル) を加えて室温下
16時間攪拌する。反応液を酢酸エチル 150 ml で希
釈し、飽和炭酸水素ナトリウム、水で順次洗浄、
乾燥後、溶媒を留去する。残液をシリカゲルカラ
ムクロマトグラフィー(溶出溶媒:ヘキサン-酢
酸エチル(3:1))で精製し、2.12 g (81%)

収:ヘキサン-酢酸エチル(1:1))で精製し、
222 mg (68%) のカラメルを得た。この化合物は
2種の立体異性体の混合物(2:1)である。

¹HMR(CDCl₃) δ

2.32(dd), 2.57(dd), 2.70(ABX type), 2.85(dd),
2.97(m), 3.11(m), 3.12(dd), 3.16(m), 3.22(dd),
3.82(br), 4.13(ABX type), 4.20(ABX type),
4.48(t), 4.53(t), 4.86~5.12(m),
7.2~7.4(m, 5H)

工程3

N-[(2, 3-ジヒドロキシ)-3-フェニ
ルプロピル]-1-デオキシノジリマイシン

N-[(2, 3-ジヒドロキシ)-3-フェニ
ルプロピル]-1-デオキシノジリマイシンテ
トラアセテート 196 mg (0.42ミリモル) をメタノ
ール 5 ml に溶解し、炭酸カリウム 3 mg を加えて室温
下3時間攪拌する。溶媒を留去した後、残液をシ
リカゲルカラムクロマトグラフィー(溶出溶媒:
クロロホルム-メタノール(3:1))で精製し
128 mg (98%) の無色カラメルを得た。この化合

物の結晶を得た。

¹HMR(CDCl₃) δ

2.01(m, 6H), 2.03(s, 3H), 2.09(s, 3H),
2.38(dd, 1H), 2.70(dt, 1H), 3.25(dd, 1H),
3.38(dd, 1H), 3.59(ddd, 1H), 4.19(dd, 1H),
4.32(dd, 1H), 4.90~5.20(m, 3H), 6.22(dt, 1H),
6.56(d, 1H), 7.15~7.50(m, 5H)

工程2

N-[(2, 3-ジヒドロキシ)-3-フェニ
ルプロピル]-1-デオキシノジリマイシンテ
トラアセテート

N-(3-フェニル-2-プロペニル)-1-
デオキシノジリマイシンテトラアセテート 305 mg
(0.70ミリモル) と N-メチルセルロリン-N-
オキシド 98 mg (0.84ミリモル) を50%アセトン 8
ml に溶解し、四酸化オスミウム 2 mg を加えて2時間
攪拌する。亜硫酸ナトリウム 250 mg、水 3 ml を加
えて1時間攪拌した後、水 30 ml で希釈し酢酸エチ
ルで抽出、水洗、乾燥後、溶媒を留去する。残液
をシリカゲルカラムクロマトグラフィー(溶出溶

物は2種の立体異性体の混合物(2:1)である。

¹HMR(CD₃COO) δ

2.05(dd), 2.17(dd), 2.23~2.35(m), 2.54(dd),
2.81(dd), 2.98(dd), 3.10(t), 3.14(t),
3.2~4.0(m), 4.50(d), 4.68(d),
7.15~7.50(m, 5H).

次に本発明のN-置換-デオキシノジリマイシ
ン誘導体の癌細胞転移抑制作用の評価結果を示す。
効果試験

試験法

マウスの腫瘍細胞であるメラノーマ B16 株より
フィドラー (Fidler) の方法 (Method in Cancer
Research, 15, 339-439, 1978) をもとに B16 高転
移株を選別し、使用した。転移抑制作用の評価は
キジマースダ (Kijima-Suda) 等の方法 (Proc.,
Natl. Acad. Sci., U.S.A., 83, 1752-1756,
1986; Cancer Research, 46, 858-862, 1986.) を
もとにして行った。まず B16 高転移株を牛胎児血
漿を加えたグルベコ ME 培地 (DME 培地) に懸
え、一般式 (1) で表される N-置換-1-デオ

キシノグリマイシンを加え、2～4日間、5%CO₂の存在下37℃で培養し、増殖した細胞をトリプシン-EDTA溶液で培養容器より剥がした。この細胞をCa⁺⁺とMg⁺⁺を含まないダルベコの平衡塩類溶液で生細胞として1ml当たり1×10⁶細胞になるように懸濁した。

この懸濁液の0.1 mlをマウス尾静脈中に注入し細胞を移植し14日間飼育した後、開腹して肺を摘出し、肺表面及び内部に形成されたB16高転移性の転移結節数を数え、薬剤処理をしなかった対照と比較した。

試験例1 細胞障害性

B16高転移性を10%牛胎児血清を加えたDME培地に5%CO₂の存在下37℃で培養し、トリプシン-EDTA溶液で培養容器より剥がし、1ml当たり1×10⁶細胞になるように懸濁した。この懸濁液の150 μlを被検薬あるいは対照薬溶液50 μlにそれぞれ加え混合した。この後、4日間培養し、倒立顕微鏡下で生死を観察し、細胞障害性を判定した。その結果を表1の通りであった。

の平衡塩類溶液で生細胞として1ml当たり1×10⁶細胞になるように懸濁し、その0.1 mlをBDF₁マウス(8週令、雄)の尾静脈に注入し、細胞を移植した。14日間飼育後、開腹して肺を摘出し、肺表面及び内部に形成されたB16高転移性の転移結節数を数えた。その結果を表2に示した。

表 2

添加薬剤	肺転移結節数(平均±標準偏差)
無添加	207±47
製造例化合物9 (30 μg/ml)	96±29
製造例化合物10 (30 μg/ml)	60±18
製造例化合物7 (30 μg/ml)	18±7

以上の結果より本発明の化合物の処理で肺に形成される転移結節数は大きく減少した。

本発明の癌細胞転移阻害剤は、上記のN-置換-1-デオキシノグリマイシン誘導体を含有する経口、経口製剤とし臨床的に肺腫、動脈、皮膚、皮下、皮内、直腸及び筋肉内を経由又は経口にて投与される。また腫瘍に直接投与することにより、より強い効果が期待できる。投与量は投与形態、

表 1

使用細胞	B16高転移性	
添加薬剤	濃度	生育
無添加		+
製造例化合物 9	10 μg/ml	+
	30 μg/ml	+
	100 μg/ml	+
製造例化合物 10	10 μg/ml	+
	30 μg/ml	+
	10 μg/ml	+
製造例化合物 7	10 μg/ml	+
	30 μg/ml	+
	100 μg/ml	+
アドリアマイシン (対照)	0.1 μg/ml	-

表中+は生育、-は死滅を表す。

以上の試験結果より本発明の化合物はB16高転移性に対して細胞障害性を示さなかった。

試験例2 抗転移作用

B16高転移性を10%牛胎児血清を加えたDME培地に植え、被検薬を1ml当たりそれぞれ30 μg加え、5%CO₂の存在下37℃で3日間培養した。試験例1と同様の方法で細胞を培養容器より剥がした。この細胞をCa⁺⁺とMg⁺⁺を含まないダルベコ

培地あるいは患者の年齢、体重、病態により異なるが、概ね1日100～3000mgを1回又は数回投与する。

経口製剤としては、錠剤の水性又は非水性溶媒剤あるいは乳剤が挙げられる。非水性の溶媒剤又は乳剤の基剤としては、プロピレングリコール、ポリエチレングリコール、グリセリン、オリーブ油、とうもろこし油、オレイン酸エチル等が挙げられる。

また、経口剤としては、カプセル剤、錠剤、咀嚼剤、散剤等が挙げられる。

これらの製剤に賦形剤として、澱粉、乳糖、マンニット、エチルセルロース、ナトリウムカルボキシメチルセルロース等が配合され、滑沢剤としてステアリン酸マグネシウム又はステアリン酸カルシウムを添加する。結合剤としては、ゼラチン、アラビアゴム、セルロースエステル、ポリビニルピロリドン等が用いられる。

次に本発明の製剤例について説明する。

〔実施例〕

である。

N-〔3-(4-フロロフェニル)-2-プロペニル〕-1-

デオキシノジリマイシン 200 mg

乳糖 130 mg

ジャガイモ澱粉 70 mg

ポリビニルピロリドン 10 mg

ステアリン酸マグネシウム 2.5 mg

乳糖及びジャガイモ澱粉を混合し、これにポリビニルピロリドンの20%エタノール溶液を加え、均一に混濁させ、100の細目のふるいを通し、45℃にて乾燥させ、再度100の細目のふるいを通した。こうして得られた顆粒をステアリン酸マグネシウムと混合し錠剤に成型した。

〔発明の効果〕

本発明は癌細胞転移抑制作用を有する極めて有用な物質である。そして、この物質を有効成分とした癌細胞転移抑制剤は、現在この防止手段が殆ど無く、癌治療者の予後を左右する最大の問題である癌細胞の転移を解決した極めて有用な発明

特許出願人

明治製菓株式会社

代理人

小 堀 益 (ほか1名)

第1頁の続き

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手 続 補 正 書

平成元年10月27日

特許庁長官 吉 田 文 毅 殿

1. 事件の表示

平成1年 特 許 願 第127499号

2. 発明の名称 新規N-置換-1-デオキシノジリマイシン誘導体及びそれを含有する癌細胞転移抑制剤

3. 補正をする者

事件との関係 特許出願人

氏 名 (609) 明治製菓株式会社

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氏 名 (8216) 弁理士 小 堀 益

5. 補正の対象

明細書

6. 補正の内容

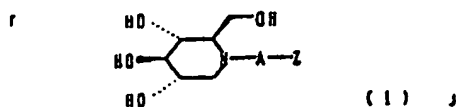


方式 ④

5 の炭化水素基を表し、この炭化水素基は二重又は三重結合を有していてもよい、Zはフェニル基、フッソ置換フェニル基、ビフェニル基、シクロアルキル基又はハロゲン置換アルキル基を表す、

で示されるN-置換-1-デオキシノジリマイシン誘導体又はその薬理的に許容される酸との付加塩を有効成分とすることを特徴とする癌細胞転移抑制剤。」

(2) 明細書第4頁の式(1)を下記の通り補正する。



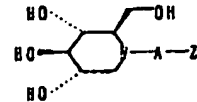
(3) 明細書第3頁第12～14行「従って、この・・・課題である。」を下記の通り補正する。

「従って、現行の癌治療の有効性は癌細胞の転移を抑制することで、さらに高められることが期待される。」

(4) 明細書第15頁下から第9行「ケニル化試剤と

(1) 特許請求の範囲を下記の通り補正する。

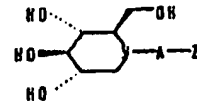
1. 式



式中、Aは水酸基、ハロゲン化アルキル基又はアルコキシ基で置換されてもよい炭素数3乃至5の炭化水素基を表し、この炭化水素基は二重又は三重結合を有していてもよい、Zはフェニル基、フッソ置換フェニル基、ビフェニル基、シクロアルキル基、又はハロゲン置換アルキル基を表す、

で示されるN-置換-1-デオキシノジリマイシン誘導体。

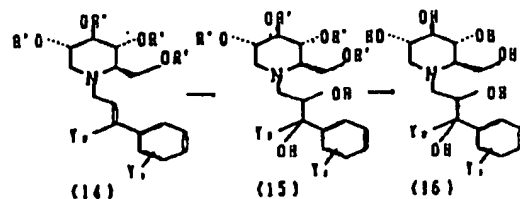
2. 式



式中、Aは水酸基、ハロゲン化アルキル基、アルコキシ基で置換されてもよい炭素数3乃至

各種アルコール類)を「ケニル化試剤と1-デオキシノジリマイシンを各種アルコール類)に補正する。

(5) 明細書第16頁の式(14)、(15)、(16)をそれぞれ下記の通り補正する。



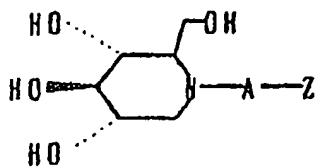
DESCRIPTION

1. TITLE OF THE INVENTION

NOVEL N-SUBSTITUTED-1-DEOXYNOJIRIMYCIN DERIVATIVE AND
CANCER CELL ANTIMETASTATIC AGENT INCLUDING THE SAME

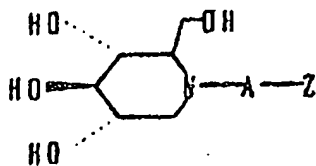
2. PATENT CLAIMS

1. An N-substituted-1-deoxynojirimycin derivative
represented by the following formula,



wherein A represents a hydrocarbon group of 3 to 5 carbon atoms optionally substituted with hydroxyl, alkyl halide or alkoxy group, the hydrocarbon group optionally comprising a double or triple bond, and Z represents phenyl, fluorinated phenyl, biphenyl, cycloalkyl or halogenated alkyl group.

2. A cancer cell antimetastatic agent characterized by an active ingredient which is an N-substituted-1-deoxynojirimycin derivative represented by the following formula or an addition salt thereof with a pharmaceutically acceptable acid,



wherein A represents a hydrocarbon group of 3 to 5 carbon atoms optionally substituted with hydroxyl, alkyl halide or alkoxy group, the hydrocarbon group optionally comprising a double or triple bond, and Z represents phenyl, fluorinated phenyl, biphenyl, cycloalkyl or halogenated alkyl group.

3. DETAILED DESCRIPTION OF THE INVENTION

[Industrial Field of Application]

The present invention relates to a novel N-substituted-1-deoxynojirimycin derivative which inhibits formation of cancer cell metastases and a cancer cell antimetastatic agent containing the same as the active ingredient.

[Conventional Technique]

Various anticancer agents are currently in use. Majority of them are drugs which kill cancer cells or let human immune system destroy them, but a drug effective for fundamental treatment of cancers has not been obtained yet.

Solid cancers, to which chemotherapeutic agents have low effectiveness, are treated with physical therapies

such as surgery or radiotherapy, and the success rate is greatly improved from a viewpoint of removing primary cancer. It is however also true that metastases of cancer cells are induced on the other side.

[Problem to be Solved by the Invention]

As described above, metastasis of cancer cells are the biggest problem in conventional cancer treatments which affects prognosis of patients with cancer.

Therefore, it is currently desired the most to develop an anticancer agent which can enhance suppression of cancer cell metastasis.

In order to achieve the above object, it is the purpose of the present invention to provide a substance which effectively suppresses cancer cell metastases and a cancer cell antimetastatic agent containing the same as the active ingredient.

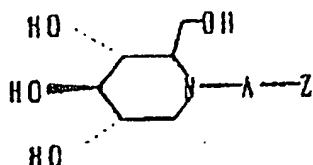
[Means for Solving the Problem]

The present inventors found N-substituted-1-deoxynojirimycin derivatives having a cancer cell antimetastatic effect prior to the present invention, and disclosed them in Japanese patent application publication Nos. Sho63-31095, Sho63-93673, Sho63-97454, Sho63-104850, Sho63-147815 and Sho63-147816.

The present inventors further synthesized novel N-

substituted derivatives of 1-deoxynojirimycin and broadly evaluated them, and then found a group of novel compounds having a strong cancer cell antimetastatic effect. The present invention has been thus accomplished.

The present invention is an N-substituted-1-deoxynojirimycin derivative represented by formula 1, and a cancer cell antimetastatic agent containing the compound or the addition salt thereof with a pharmaceutically acceptable acid as the active ingredient,



(1)

wherein A represents a hydrocarbon group of 3 to 5 carbon atoms optionally substituted with hydroxyl, alkyl halide or alkoxy group, the hydrocarbon group optionally comprising a double or triple bond, and Z represents phenyl, fluorinated phenyl, biphenyl, cycloalkyl or halogenated alkyl group.

The N-substituted-1-deoxynojirimycin derivative shown by formula 1 of the present invention is a novel substance which has not ever described in documents.

The following substances are examples of the compounds included in the novel N-substituted-1-deoxynojirimycin derivative:

N-(3-methoxymethyl-3-phenyl-2-propeny)-1-

deoxynojirimycin,
 N-(3-phenyl-3-trifluoromethyl-2-propenyl)-1-
 deoxynojirimycin,
 N-[3-(4-fluorophenyl)-2-propenyl]-1-deoxynojirimycin,
 N-[3[(3-fluorophenyl)-2-propenyl]-1-deoxynojirimycin,
 N-[3[(2-fluorophenyl)-2-propenyl]-1-deoxynojirimycin,
 N-[3-(4-biphenylpropyl)]-1-deoxynojirimycin,
 N-[3-(4-fluorophenyl)-propyl]-1-deoxynojirimycin,
 N-(3-cyclohexylpropyl)-1-deoxynojirimycin,
 N-(3-phenyl-2-propnlyl)-1-deoxynojirimycin,
 N-(2,3-dihydroxy-3-phenylpropenyl)-1-deoxynojirimycin,
 N-(6,6,6-trifluorohexyl)-1-deoxynojirimycin,
 N-(5,5,5-trifluoropentyl)-1-deoxynojirimycin, and
 N-(4,4,4-trifluorobutyl)-1-deoxynojirimycin.

When the N-substituted-1-deoxynojirimycin derivative
 of the present invention is used as a cancer cell
 antimetastatic agent, the pharmaceutically acceptable acid
 addition salt thereof includes addition salts of:
 inorganic acids such as hydrochloric acid, hydrobromic
 acid, sulfuric acid, nitric acid and phosphoric acid;
 organic acids such as formic acid, acetic acid, propionic
 acid, succinic acid, glycolic acid, lactic acid, malic
 acid, tartaric acid, citric acid, maleic acid, fumaric
 acid, benzoic acid, salicylic acid and methanesulfonic
 acid; and also amino acids such as asparaginic acid and
 glutamic acid.

All compounds of the present invention are novel compounds which have not ever described in documents. According to the most general synthesis method thereof, 1-deoxynojirimycin (see Tetrahedron, 24, 2125(1968)) is used as the raw material, which is obtained by reducing nojirimycin-(5-amino-5-deoxy-D-glucopyranose) (see Japanese patent application publication No. Sho43-760) which is a metabolite of an actinomycete found by the present inventors. Specifically, the N-substituted A-Z group of formula 1 of the present invention may be introduced by heating or leaving at room temperature 1-deoxynojirimycin with an aralkyl- or aralkenylation agent typrified by aralkyl halide or alkenyl halide, aralkylsulfonate ester or aralkenylsulfonate ester, etc. in polar solvent such as alcohols, dimethylformamide, dimethylacetoamide, dimethylsulfoxide, sulfolane and the mixture thereof in the presence of a deoxidizing agent such as alkali hydroxide, alkali carbonate, alkali bicarbonate, suitable organic amines, etc. It is also possible to employ a method such that the raw material is 1-deoxynojirimycin whose hydroxyl group is protected by a suitable protecting group, for example acetyl, benzoyl, tetrahydropyranyl, t-butyldimethylsilyl, or the like, and is subjected to the N-substitution reaction followed by deprotection. Furthermore, also available are: a method to carry out so-called reductive alkylation by use of an

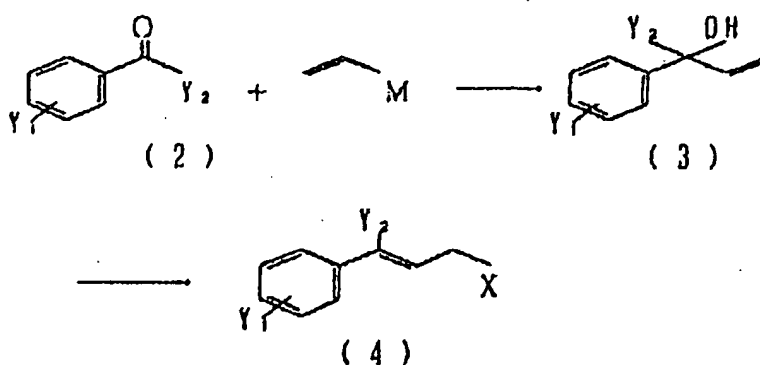
agent with carbonyl group as an reactive agent in hydrogen atmosphere under a reductive condition, for example conditions in the presence of formic acid, sodium cyanoborohydride, sodium borohydride or a suitable metal catalyst of platinum oxide, palladium or Raney nickel; and a method to obtain an objective product by reducing an amide compound of 1-deoxynojirimycin with aralkylcarbonic acid or aralkenylcarbonic acid. According to need, these compounds are subjected to a general purification procedure such as recrystallization, column chromatography, etc., so as to obtain the compound of formula 1 of the present invention.

The substitution group of the compound of the present invention may be formed and introduced by any method suitable for the purpose. The following five production methods are given as suitable methods to produce an aralkyl-, aralkenyl- or aralkynylation agent for constructing the A-Z group of formula 1.

[Production Method 1]

Compound 3 may be synthesized by the reaction of compound 2 with a vinyl-metal compound, for example vinylmagnesium chloride, divinylmagnesium bromide, vinylmagnesium iodide, vinyl lithium, divinylzinc, divinylcopper, divinylcesium, or the like, in nonpolar solvent, preferably in ether, tetrahydrofuran or dioxane, at -50°C to room temperature for 10 minutes to 24 hours.

Compound 4 may be synthesized by the reaction of compound 3 with hydrochloric acid, hydrobromic acid, oxalyl chloride, thionyl halide, oxyphosphorus halide, phosphorus trihalide, phosphorus pentahalide, tri-substituted phosphine-carbon tetrahalide, allyl- or alkylsulfonyl halide without solvent or in solvent such as benzene, toluene, ether, methylene chloride, acetonitrile, etc. at 0°C to 100°C for 30 minutes to 24 hours, the reaction being accompanied with transfer of the allylalcohol part of compound 3.



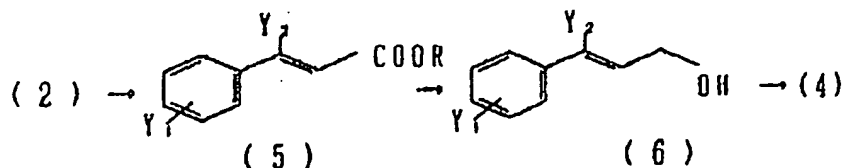
In the formula, Y₁ represents hydrogen atom, halogen atom, aralkyl or hydroxyl group, Y₂ represents hydrogen atom, halogen atom, aralkyl, alkoxy or halogen-substituted alkyl group, X represents halogen atom or alkyl- or allylsulfonyloxy group. The halogen atom denotes chlorine, brome, iodine atom, etc., and the alkyl- or allylsulfonyloxy group denotes methane sulfonyloxy, trifluoromethane sulfonyloxy, p-toluene sulfonyloxy group, etc. M represents mono- or divalent metal or the salt

thereof, and the metal denotes lithium, sodium, potassium, magnesium, zinc, cesium or copper.

[Production Method 2]

Unsaturated ester 5 is synthesized by the reaction of compound 2 with carboalkoxymethylene tri-substituted phosphorane in suitable solvent, preferably benzene, toluene, ether, tetrahydrofuran, dioxane, methylene chloride, chloroform, methanol and ethanol, at 0°C to 60°C for 10 minutes to 24 hours, or with diaralkylphosphonoacetic acid aralkylester in the presence of a suitable base, for example sodium hydride, potassium hydride, alkali hydride or alkali carbonate, at 0°C to 60°C for 10 minutes to 24 hours. Compound 6 may be synthesized by the reaction of compound 5 with a suitable metal hydride complex reductant, preferably lithium aluminum hydride, diisobutylaluminum hydride, sodium bis(2-methoxyethoxy)aluminum hydride, or the like, in suitable aprotic solvent, preferably ether, tetrahydrofuran or dioxane, at -78°C to -100°C for 30 minutes to 18 hours. Compound 4 may be synthesized by the reaction of compound 6 with hydrochloric acid, hydrobromic acid, oxalyl chloride, thionyl halide, phosphorus trihalide, phosphorus pentahalide, tri-substituted phosphine-carbon tetrahalide, allyl- or alkylsulfonyl halide without solvent or in solvent such as benzene, toluene, ether, methylene chloride, acetonitrile etc. at 0°C to 100°C for 30 minutes

to 24 hours.

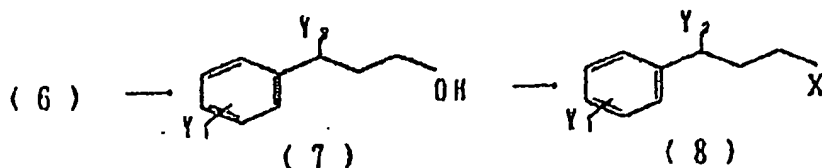


In the formula, Y_1 and Y_2 represent the same as above, and R represents a protection group of carboxyl such as alkyl.

[Production Method 3]

Saturated alcohol 7 may be synthesized by the reduction of alkenylalcohol 6 obtained in production method 2 in the presence of a metal catalyst, for example palladium-carbon, platinum, Raney nickel, or the like, in suitable organic solvent, for example methanol, ethanol, acetic acid, tetrahydrofuran, ethyl acetate, or the like, in hydrogen atmosphere for 30 minutes to 24 hours.

Compound 8 may be synthesized by the reaction of compound 7 in solvent such as hydrobromic acid, oxalyl chloride, thionyl halide, phosphorous oxyhalide, phosphorous trihalide, phosphorous pentahalide, tri-substituted phosphine-carbon tetrahalide, allyl- or alkylsulfonyl halide, etc. at 0°C to 100°C for 30 minutes to 24 hours.

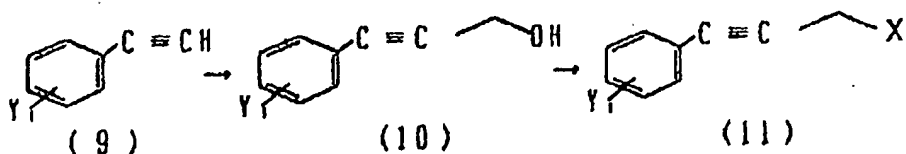


In the formula, Y_1 , Y_2 and X represent the same as

above.

[Production Method 4]

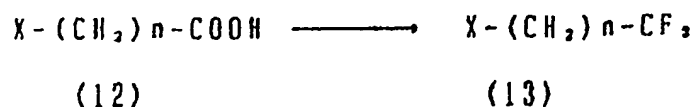
Alkynylalcohol 10 may be synthesized by acetylidation of 1-allylacetylene derivative 9 with a suitable base, for example n-butyllithium, lithium diisopropylamide, sodium amide or the like, followed by reaction with formalin. Compound 11 may be synthesized by the reaction of compound 10 with oxalyl chloride, thionyl halide, phosphorous oxyhalide, phosphorous trihalide, phosphorous pentahalide, tri-substituted phosphine-carbon tetrahalide or allyl- or alkylsulfonyl halide without solvent or in solvent such as benzene, toluene, ether, methylene chloride, acetonitrile, etc. at 0°C to 100°C for 30 minutes to 24 hours.



In the formula, Y1, Y2 and X represent the same as above.

[Production Method 5]

As a production method of a terminally halogenated alkylation agent, for example, a trifluoromethyl derivative 13 may be synthesized by treating ω -halogenated fatty acid 12 with a suitable fluorinating agent, for example sulfur tetrafluoride (Angew. Chem. Internat. Ed., 1, 467(1962)).



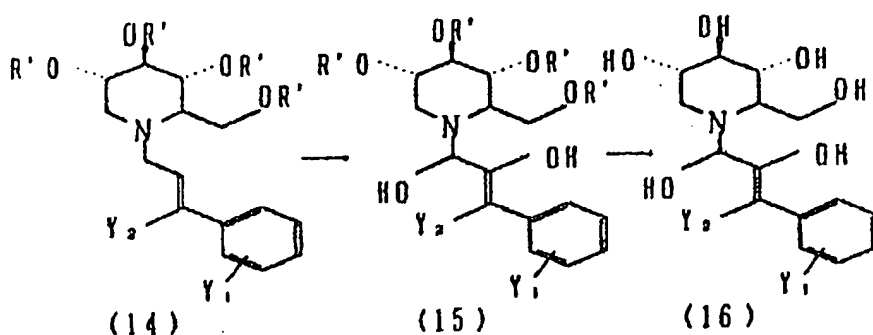
In the formula, X represents the same as above.

The N-substituted A-Z group of the compound of formula 1 in the present invention may be introduced by heating or leaving at room temperature with an aralkyl- or aralkenylation agent typified by the aralkyl halide or aralkenyl halide produced by the above production methods 1 to 5 and aralkylsulfonate ester or aralkenylsulfonate ester in polar solvent such as alcohols, dimethylformamide, dimethylacetoamide, dimethylsulfoxide, sulfolane, etc. or the mixture thereof in the presence of a deoxidizing agent such as alkali hydroxide, alkali carbonate, alkali bicarbonate or suitable organic amines. It is also possible to employ a method such that the raw material is 1-deoxynojirimycin whose hydroxyl is protected by a suitable protecting group, for example acetyl, benzoyl, tetrahydropyranyl, t-butyldimethylsilyl, or the like, and N-substitution reaction is carried out followed by deprotection. Among the compounds included in the present invention, the ones of formula 1 where A is a hydroxyl-substituted hydrocarbon may be produced according to the following production method 6.

[Production method 6]

Objective product 16 may be obtained by the reaction

of N-substituted-1-deoxynojirimycin derivative 14, which may be synthesized by the reaction of the alkenylation agent synthesized according to production method 1 or 2 with 1-deoxynojirimycin or 1-deoxynojirimycin with protected hydroxyl, with a suitable oxidization agent, for example osmium tetroxide, or the like.



In the formula, Y₁ and Y₂ represent the same as above, R' represents hydrogen atom, acetyl, benzil, benzoyl, pivaloyl, t-butyldimethylsilyl or tetrahydropyranyl group.

Next, production examples of the N-substituted-1-deoxynojirimycin derivative of the present invention are shown.

[Production Example 1]:

N-(3-phenyl-3-trifluoromethyl-2-propenyl)-1-deoxynojirimycin

[Step 1]:

3-phenyl-3-trifluoromethyl-2-propene-1-ol

A solution of 1.74 g (10.0 mmol) 2,2,2-trifluoroacetophenone, which was dissolved in 10 ml of

tetrahydrofuran, was cooled to -78°C , and 1M vinylmagnesiumbromide solution in tetrahydrofuran was added dropwise. Following to the addition, the solution was stirred for 3 hours, and further for 1 hour without the cool bath. Water was added to decompose excess reagent in ice bath, and the solvent was then distilled away. 10 ml of 2N sulfuric acid was added to the residue, and extraction was carried out with ethyl acetate. The extract was washed with water, dried and then concentrated. The residue was purified with silica gel column chromatography (eluting solvent: ether-hexane (1:10)), so as to obtain 1.66 g (82%) of oily product.

NMR (CDCl_3) δ

2.61 (s, 1H), 5.52 (d, 1H), 5.62 (d, 1H),
6.43 (dd, 1H), 7.25-7.70 (m, 5H)

[Step 2]:

1-bromo-3-phenyl-3-trifluoromethyl-2-propene

606 mg (3.00 mmol) of 3-phenyl-3-trifluoromethyl-2-propene-1-ol and 943 mg (3.60 mmol) of triphenylphosphine were dissolved in 4 ml of acetonitrile and cooled in ice bath. 1.26 g (3.80 mmol) of carbon tetrabromide was then added in several parts. The solution was stirred for 1 hour in ice bath, and then further stirred overnight at room temperature. The reaction was diluted with 10 ml of ether, deposited solid was filtered off, and the filtrate was concentrated. The obtained residue was purified with

silica gel column chromatography (eluting solvent: hexane), so as to obtain 440 mg (55%) of oily product.

NMR (CDCl₃) δ

3.80 (dq, 2H), 8.62 (tq, 1H), 7.20-7.60 (m, 5H)

[Step 3]:

N-(3-phenyl-3-trifluoromethyl-2-propenyl)-1-deoxynojirimycin

163 mg (1.00 mmol) of deoxynojirimycin and 318 mg (1.20 mmol) of 1-bromo-3-phenyl-3-trifluoromethyl-2-propene were dissolved in 5 ml of dimethylformamide. 207 mg (1.50 mmol) of potassium carbonate was added and the solution was stirred for 8 hours at room temperature. Saturated salt solution was added to the reaction mixture, and extraction was carried out with n-butanol. The extract was concentrated under reduced pressure, and the residue was purified with silica gel column chromatography (eluting solvent: chloroform-methanol (10:1)), so as to obtain 311 mg (90%) of colorless solid product.

NMR (CD₃OD) δ

2.15 (m, 2H), 3.10 (dd, 1H), 3.16 (t, 1H),

3.31 (m, 1H), 3.42 (t, 1H), 3.53 (m, 1H),

3.78 (dd, 1H), 3.96 (ABX type, 2H),

6.72 (t, 1H), 7.32 (m, 2H), 7.46 (m, 3H)

[Production Example 2]:

N-(3-metoxymethyl-3-phenyl-2-propenyl)-1-deoxynojirimycin

The synthesis was carried out by use of 1-bromo-3-

metoxymethyl-3-phenyl-2-propene which was synthesized in the same manner as production method 1.

NMR (CD₃OD) δ

2.13 (m, 2H), 3.06 (dd, 1H), 3.16 (t, 1H),

3.34 (m, 1H), 3.44 (t, 1H), 3.31 (m, 1H),

3.38 (s, 3H), 3.76 (dd, 1H),

3.97 (ABX type, 2H), 4.16 (s, 2H),

6.06 (t, 1H), 7.15-7.50 (m, 5H)

[Production example 3]:

N-[3-(4-fluorophenyl)-2-propenyl]-1-deoxynojirimycin

[Step 1]:

Methyl-3-(4-fluorophenyl)-2-propenoate

1.24 g (10.0 mmol) of 4-fluorobenzaldehyde was dissolved in 20 ml of methylene chloride. 3.67 g (11.0 mmol) of carbomethoxymethylenetriphenylphosphorane was added, and the mixture was stirred for 3 hours at room temperature. Solid was filtered off, the filtrate was concentrated, and the residue was purified with silica gel chromatography (eluting solvent: ethyl acetate-hexane (1:4)), so as to obtain 1.61 g (90%) of colorless needle crystal.

NMR (CDCl₃) δ

4.30 (d, 2H), 6.25 (m, 1H), 6.55 (d, 1H),

6.95 (m, 2H), 7.35 (m, 2H)

[Step 2]:

3-(4-fluorophenyl)-2-propene-1-ol)

1.61 g (9.00 mmol) of methyl-3-(4-fluorophenyl)2-propenoate was dissolved to 50 ml of ether, and the solution was dropwise added to 205 mg (5.40 mmol) of lithium aluminum hydride suspended in 3 ml of ether in ice bath. Stirring for 30 min at room temperature after the addition, excess reagent was then decomposed with water, and solid was filtered off. The filtrate was concentrated, so as to obtain 1.33 g (97%) of 3-(4-fluorophenyl)-2-propene-1-ol.

NMR (CDCl₃) δ

4.52 (d, 2H), 6.31 (m, 1H), 7.01 (m, 2H),

7.45 (m, 2H)

[Step 3]:

1-bromo-3-(4-fluorophenyl)-2-propene

1.34 g (8.82 mmol) of 3-(4-fluorophenyl)-2-propene-1-ol and 4.26 g (11.5 mmol) of tri-n-octylphosphine was dissolved in 20 ml of ether, and 3.52 g (10.6 mmol) of carbon tetrabromide was added in several parts in ice bath. After stirring for 30 min at room temperature, precipitate was filtered off, the filtrate was concentrated, and the residue was purified with silica gel column chromatography (eluting solvent: hexane), so as to obtain 1.61 g (85%) of colorless oily product.

NMR (CDCl₃) δ

3.35 (d, 2H), 6.30 (m, 1H), 7.00 (m, 2H),

7.40 (m, 2H)

Mass m/z 214, 216

[Step 4]:

N-[3-(4-fluorophenyl)-2-propenyl]-1-deoxynojirimycin

1.61 g (7.5 mmol) of 1-bromo-3-(4-fluorophenyl)-2-propene and 1.22 g (7.5 mmol) of 1-deoxynojirimycin were dissolved in 10 ml of dimethylformamide. 3.12 g (22.5 mmol) of Potassium carbonate was added and stirred 24 hours at room temperature. Water was added to the reaction mixture, and extraction was carried out with n-butanol. After distilling away the solvent, the residue was purified with silica gel column chromatography (eluting solvent: chloroform-methanol (10:1)), so as to obtain 1.36 g (61%) of pale yellow solid product.

NMR (CD₃OD) δ

2.4-4.2 (m, 16H), 6.40 (m, 1H), 6.7 (m, 1H),

7.10 (m, 2H), 7.55 (m, 2H)

Mass m/z 298 (FD, M+1)

[Production Example 4]:

N-[3-(3-fluorophenyl)-2-propenyl]-1-deoxynojirimycin

The synthesis was carried out in the same manner as production example 3.

NMR (CD₃OD) δ

2.15 (m, 2H), 3.04 (dd, 1H), 3.14 (t, 1H),

3.2-3.35 (m, 1H), 3.39 (t, 1H),

3.49 (m, 1H), 3.68 (dd, 1H),

3.94 (ABX type, 2H), 6.41 (dt, 1H),

6.59 (d, 1H), 6.95 (dt, 1H), 7.16 (dd, 1H),
7.21 (d, 1H), 7.31 (ddd, 1H)

Mass m/z 298 (FD, M+1)

[Production Example 5]:

N-[3-(2-fluorophenyl)-2-propenyl]-1-deoxynojirimycin

The synthesis was carried out in the same manner as production example 3.

NMR (CD₃OD) δ

2.1-2.25 (m, 2H), 3.06 (dd, 1H),
3.14 (t, 1H), 3.24-3.35 (m, 1H),
3.39 (t, 1H), 3.50 (m, 1H), 3.71 (m, 1H),
3.94 (ABX type, 2H), 6.45 (dt, 1H),
6.72 (d, 1H), 7.0-7.16 (m, 2H),
7.2-7.28 (m, 1H), 7.53 (dt, 1H)

Mass m/z (FD, M+1)

[Production Example 6]:

N-[3-(4-biphenyl)propyl]-1-deoxynojirimycin

[Step 1]:

1.10 g (6.00 mmol) of methyl-3-(4-biphenyl)acrylate-4-biphenylcarboxyaldehyde was dissolved in 20 ml of dichloroethane. 3.03 g (9.10 mmol) of carbomethoxymethylenetriphenylphosphorane was added, and the solution was stirred for 1 hour at room temperature. After distilling away the solvent, the residue was purified with silica gel column chromatography (eluting solvent: ether-hexane (1:10)), so as to obtain 1.12 g

(78%) of colorless crystal.

NMR (CDCl₃) δ

3.83 (s, 3H), 6.49 (d, 1H), 7.30-7.60 (m, 9H),

7.75 (d, 1H)

[Step 2]:

Methyl-3-(4-biphenyl)propionate

1.40 g (4.40 mmol) of methyl-3-(4-biphenyl)acrylate was dissolved in 50 ml of ethyl acetate. 70 mg of 10% Pd-C was added to carry out catalytic reduction under ambient pressure for 12 hours. After filtering off the catalyst, the solvent was distilled away so as to obtain 1.01 g (97%) of colorless oily product.

NMR (CDCl₃) δ

2.68 (t, 2H), 3.00 (t, 2H), 3.68 (s, 3H),

7.20-7.70 (m, 9H)

[Step 3]:

3'-(4-biphenyl)-1-propanol

To suspension of 110 mg (2.90 mmol) lithium aluminum hydride in 10 ml of ether, solution of 1.01 g (4.20 mmol) of methyl-3-(4-biphenyl)propionate in 35 ml of ether was added dropwise in ice bath. After stirring for 1 hour at the same temperature, excess reagent was decomposed with water, inorganic product was filtered off, and the filtrate was dried and concentrated, so as to obtain 861 mg (96%) of colorless crystal.

NMR (CDCl₃) δ

1.56 (br, 1H), 1.94 (m, 2H), 2.77 (m, 2H),
3.71 (m, 2H), 7.15-7.76 (m, 9H)

[Step 4]:

3-(4-biphenyl)-1-bromopropane

419 mg (2.00 mmol) of 3-(4-biphenyl)-1-propanol and 629 mg (2.40 mmol) of triphenylphosphine was dissolved in 10 ml of ether. 930 mg (2.80 mmol) of carbon tetrabromide was added in ice bath in several parts. After stirring for 1 hour at room temperature, precipitate was filtered off, the filtrate was concentrated, and the residue was purified with silica gel column chromatography (eluting solvent: hexane), so as to obtain 506 mg (92%) of colorless oily product.

NMR (CDCl₃) δ

2.20 (quin, 2H), 2.83 (t, 2H), 3.44 (t, 2H),
7.23-7.65 (m, 9H)

[Step 5]:

N-[3-(4-biphenyl)propyl]-1-deoxynojirimycin

140 mg (0.50 mmol) of 3-(4-biphenyl)-1-bromopropane and 82 mmol (0.5 mmol) of 1-deoxynojirimycin were dissolved in 1 ml of dimethylformamide. 136 mg (1.00 mmol) of potassium carbonate was added and heated at 80°C for 4 hours. Water was added, and the reaction mixture was acidified with hydrogen chloride and washed with ether. The aqueous phase was alkalized with ammonia, and extraction was carried out with n-butanol. After removing

the solvent, the residue was purified with silica gel column chromatography (eluting solvent: chloroform-methanol (10:1), so as to obtain 117 mg (66%) of solid product.

NMR (CD₃OD) δ

1.86 (m, 2H), 2.20 (br, 2H), 2.65 (m, 3H),
2.89 (m, 1H), 3.00 (m, 1H), 3.14 (t, 1H),
3.47 (m, 1H), 3.84 (d, 2H), 7.15-7.65 (m, 9H)

[Production Example 7]:

N-[3-(4-fluorophenylpropyl)]-1-deoxynojirimycin

The synthesis was carried out in the same manner as production example 6.

NMR (CD₃OD) δ

1.38 (m, 2H), 2.05-2.22 (m, 2H), 2.64 (m, 2H)
2.98 (dd, 1H), 3.13 (t, 1H), 3.30 (m, 1H),
3.38 (t, 1H), 3.45 (m, 1H),
3.64 (m, 1H), 3.85 (m, 2H), 7.18-7.35 (m, 4H)

[Production Example 8]

N-(3-cyclohexylpropyl)-1-deoxynojirimycin

The synthesis was carried out with the same manner as production example 6.

NMR (CD₃OD) δ

0.75-1.08 (m, 2H), 1.08-1.45 (m, 7H),
1.45-2.00 (m, 6H), 2.70-3.83 (m, 8H),
4.00 (ABX type, 2H)

[Production Example 9]:

N-(phenyl-2-propynyl)-1-deoxynojirimycin

[Step 1]:

1-phenyl-3-bromopropin

660 mg (5.00 mmol) of 1-phenyl-2-propin-1-ol and 4.98 g (15.0 mmol) of carbon tetrabromide were dissolved in 30 ml of tetrahydrofuran. 2.62 g (10.0 mmol) of triphenylphosphine was added thereto in ice bath in several parts. After stirring for 10 hours at room temperature, solid was filtered off and the filtrate was concentrated. The residue was purified with silica gel column chromatography (eluting solvent: hexane), so as to 181 mg (65%) of colorless oily product.

NMR (CDCl₃) δ

1.20 (br, 1H), 2.27 (s, 1H), 7.15-7.40 (m, 5H)

[Step 2]:

N-(phenyl-2-propynyl)-1-deoxynojirimycin

163 mg (1.00 mmol) of 1-deoxynojirimycin and 215 mg (1.10 mmol) of 1-phenyl-3-bromopropyne were dissolved in 3 ml of dimethylformamide. 166 mg (1.20 mmol) of potassium carbonate was added thereto and stirred for 8 hours at room temperature. Water was added, and the reaction mixture was acidified with hydrogen chloride and washed with ether. The aqueous phase was alkalized with ammonia, and extraction was carried out with n-butanol. After distilling away the solvent, the residue was purified with silica gel column chromatography (eluting solvent:

chloroform-methanol (10:1)), so as to obtain 181 mg (65%) of solid product.

NMR (CD₃OD) δ

2.31 (d, 1H), 2.57 (t, 1H), 2.98 (dd, 1H),

3.19 (t, 1H), 3.50 (t, 1H), 3.61 (m, 1H),

3.82 (ABX type, 2H), 3.98 (dd, 2H)

[Production Example 10]:

N-[(2,3-dihydroxy)-3-phenylpropyl]-1-deoxynojirimycin

[Step 1]:

N-(3-phenyl-2-propenyl)-1-deoxynojirimycin tetraacetate

1.42 g (7.20 mmol) of cinnamylbromide and 978 mg (6.00 mmol) of 1-deoxynojirimycin were suspended in 10 ml of dimethylformamide. 996 mg (7.20 mmol) of Potassium carbonate was added and heated at 60 to 65°C for 4 hours. After cooled, the mixture was diluted with 3 ml of methylene chloride. 3.06 g (30.0 mmol) of acetic anhydride and 2.37 g (30.0 mmol) of pyridine were added and stirred for 16 hours at room temperature. The reaction was diluted with 150 ml of ethyl acetate, washed with saturated sodium hydrogen carbonate solution and subsequently with water. After dried, the solvent was then distilled away. The residue was purified with silica gel column chromatography (eluting solvent: hexane-ethyl acetate (3:1)), so as to obtain 2.12 g (81%) of crystal.

NMR (CDCl₃) δ

2.01 (s, 6H), 2.03 (s, 3H), 2.09 (s, 3H),

2.38 (dd, 1H), 2.70 (dt, 1H), 3.25 (dd, 1H),
 3.38 (dd, 1H), 3.59 (ddd, 1H), 4.19 (dd, 1H),
 4.32 (dd, 1H), 4.90-5.20 (m, 3H), 6.22 (dt, 1H),
 6.56 (d, 1H), 7.15-7.50 (m, 5H)

[Step 2]:

N-[(2,3-dihydroxy)-3-phenylpropyl]-1-deoxynojirimycin
 tetraacetate

305 mg (0.70 mmol) of N-(3-phenyl-2-propenyl)-1-deoxynojirimycin tetraacetate and 98 mg (0.84 mmol) of N-methylmorpholine-N-oxide were dissolved in 8 ml of 50% acetone. 2 mg of osmium tetroxide was added and stirred for 2 hours. After adding 250 mg of sodium nitrite and 3 ml of water and stirring for 1 hours, the solution was diluted with 30 ml of water and extraction was carried out with ethyl acetate. After washed with water and dried, the solvent was distilled away. The residue was purified with silica gel column chromatography (eluting solvent: hexane-ethyl acetate (1:1)), so as to obtain 222 mg (68%) of caramel product. This compound was a mixture (2:1) of two stereoisomers.

NMR (CDCl₃) δ

2.32 (dd), 2.57 (dd), 2.70 (ABX type), 2.85 (dd),
 2.97 (m), 3.11 (s), 3.12 (dd), 3.16 (s), 3.22 (dd),
 3.82 (br), 4.13 (ABX type), 4.20 (ABX type),
 4.48 (t), 4.53 (t), 4.86-5.12 (m),
 7.2-7.4 (m, 5H)

[Step 3]:

N-[(2,3-dihydroxy)-3-phenylpropyl]-1-deoxynojirimycin

196 mg (0.42 mmol) of N-[(2,3-dihydroxy)-3-phenylpropyl]-1-deoxynojirimycin tetraacetate was dissolved in 5 ml of methanol. 3 mg of potassium carbonate was added and stirred for 3 hours at room temperature. After distilling away the solvent, the residue was purified with silica gel column chromatography (eluting solvent: chloroform-methanol (3:1)), so as to obtain 128 mg (98%) of colorless caramel product. This compound was a mixture (2:1) of two stereoisomers.

NMR (CD₃OD) δ

2.05 (dd), 2.17 (dd), 2.23-2.35 (m), 2.54 (dd),
2.87 (dd), 2.98 (dd), 3.10 (t), 3.14 (t),
3.2-4.0 (m), 4.50 (d), 4.68 (d),
7.15-7.50 (m, 5H).

Next, shown are results of evaluating cancer cell antimetastatic effect of the N-substituted deoxynojirimycin derivatives of the present invention.

[Effect Test]

[Test Method]

From melanoma B16 strain, which is a mouse tumor cell, a B16 high metastatic strain was selected for use based on the Fidler's method (Method in Cancer Research, 15, 339-439, 1978). Antimetastatic effect was evaluated based on the method of Kijima-Suda and others (Proc.,

Natl., Acad., Sci., U.S.A., 83, 1752-1756, 1986; Cancer Research, 46, 858-862, 1986.). First, the B16 high metastatic strain was seeded on Dulbecco's ME medium (DME medium) containing fetal bovine serum. N-substituted-1-deoxynojirimycin represented by general formula 1 was added, and the cells were cultured for 2 to 4 days at 37°C in the presence of 5% CO₂. The grown cells were peeled from the culture vessel with trypsin-EDTA solution. These cells were suspended in Dulbecco's balanced salt solution without Ca⁺⁺ and Mg⁺⁺ at 1x10⁶ cells/1 ml based on living cells.

Mice were injected with 0.1 ml of this suspension via tale vine to transplant the cells. After grown for 14 days, the lungs were extirpated by laparotomy. The number of the surface and internal metastatic nodes of B16 high metastatic strain formed on the lungs was counted and compared with the control which was not treated with the agent.

[Test Example 1]: Cellular Cytotoxicity

The B16 high metastatic strain was cultured in DME medium containing 10% fetal bovine serum at 37°C in the presence of 5% CO₂. The cells were peeled from the culture vessel with trypsin-EDTA solution, and suspended at 1x10⁴ cells per 1 ml. 150 µl of the suspension were added to and mixed with each 50 µl of test drug and control drug solution. The cells were then cultured for 4

days, and the living/dead thereof was observed under an inverted microscope to decide cellular cytotoxicity. The result is shown in Table 1.

Table 1

Used cell	B16 high metastasis strain	
	Concentration	Viability
Non-added		+
Compound of Production Example 9	10 $\mu\text{g/ml}$	+
	30 $\mu\text{g/ml}$	+
	100 $\mu\text{g/ml}$	+
Compound of Production Example 10	10 $\mu\text{g/ml}$	+
	30 $\mu\text{g/ml}$	+
	10 $\mu\text{g/ml}$	+
Compound of Production Example 7	10 $\mu\text{g/ml}$	+
	30 $\mu\text{g/ml}$	+
	100 $\mu\text{g/ml}$	+
Adriamycin (control)	0.1 $\mu\text{g/ml}$	-

"+" represents "living" and "-" represents "dead".

According to the test result, the compounds of the present invention did not have cellular cytotoxicity to B16 high metastatic strain.

[Test Example 2]: Antimetastatic Effect

B16 high metastatic strain was seeded to DME medium containing 10% fetal bovine serum. Each test drug was added at 30 μg per 1 ml, and the cells were cultured for 3 days at 37°C in the presence of 5% CO₂. The cells were peeled from the culture vessel in the same way as test example 1. These cells were suspended in Dulbecco's

balanced salt solution without Ca^{++} and Mg^{++} at 1×10^6 cells/1 ml based on living cells. BDF₁ Mice (8 weeks old, male) were injected with 0.1 ml thereof via tail vein to transplant the cells. After grown for 14 days, the lungs were extirpated by laparotomy. The number of the surface and internal metastatic nodes of B16 high metastatic strain formed in the lungs was counted. The result is shown in Table 2.

Table 2

Added drug	The number of lung metastatic nodes (average \pm standard deviation)
Non-added	207 \pm 47
Compound of Production Example 9 (30 $\mu\text{g/ml}$)	96 \pm 29
Compound of Production Example 10 (30 $\mu\text{g/ml}$)	60 \pm 18
Compound of Production Example 7 (30 $\mu\text{g/ml}$)	18 \pm 7

According to the result, the treatment with the compounds of the present invention greatly reduced the number of metastatic nodes formed in the lung.

The cancer cell antimetastatic agent of the present invention is oral or parenteral formulate containing the above N-substitued-1-deoxynojirimycin derivative, and clinically administered via vein, artery, skin, subcutaneous, intracutaneous, rectum or muscle, or orally. It is expected that direct administration to a tumor brings intense effect. The dose, which depends on

administration route, dosage form, and age, weight and condition of a patient, is basically 100 to 3,000 mg per day and given one or several times.

As the parenteral formulate, there can be given sterile aqueous and non-aqueous liquid formulation and emulsion formulation. As the base of the non-aqueous liquid formulation and emulsion formulation, there can be given propylene glycol, polyethylene glycol, glycerin, olive oil, corn oil, ethyl oleate, etc.

As the oral formulate, there can be given capsule, tablet, granule, powder, etc.

To these formulates, starch, lactose, mannite, ethylcellulose, sodium carboxymethylcellulose or the like is blended as excipient, and magnesium stearate or calcium stearate is added as lubricant. As binder, gelatin, gum arabic, cellulose ester, polyvinylpyrrolidone or the like is used.

Next, a formulation example of the present invention is described.

[Example]

N-[3-(4-fluorophenyl)-2-propenyl]-1-deoxynojirimycin: 200 mg

lactose: 130 mg

potato starch: 70 mg

polyvinylpyrrolidone: 10 mg

magnesium stearate: 2.5 mg

Lactose and potato starch were mixed and wetted uniformly with 20% solution of polyvinylpyrrolidone in ethanol. The mixture was filtered with 1 mm mesh, dried at 45°C, and filtered with 1 mm mesh again. The obtained granule was mixed with magnesium stearate, and shaped to tablets.

[Advantage of the Invention]

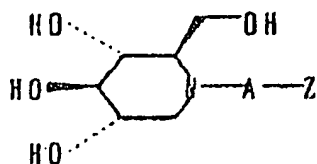
The present invention is a highly useful substance having cancer cell antimetastatic effect. The cancer cell antimetastatic agent containing this substance as the active ingredient solves the problem of cancer cell metastasis, which there is currently little countermeasure for and affects prognosis of patients with cancer the most, and is therefore a highly useful invention.

AMENDMENT

6. Content of Amendment

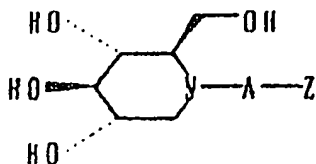
(1) The patent claims are amended as follows.

"1. An N-substituted-1-deoxynojirimycin derivative represented by the following formula,



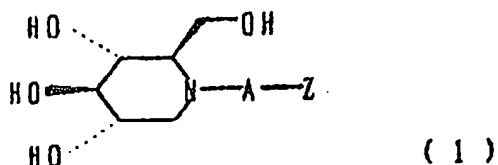
wherein A represents a hydrocarbon group of 3 to 5 carbon atoms optionally substituted with hydroxyl, alkyl halide or alkoxy group, the hydrocarbon group optionally comprising a double or triple bond, and Z represents phenyl, fluorinated phenyl, biphenyl, cycloalkyl or halogenated alkyl group.

2. A cancer cell antimetastatic agent characterized by an active ingredient which is an N-substituted-1-deoxynojirimycin derivative represented by the following formula or an addition salt thereof with a pharmaceutically acceptable acid,



wherein A represents a hydrocarbon group of 3 to 5 carbon atoms optionally substituted with hydroxyl, alkyl halide or alkoxy group, the hydrocarbon group optionally comprising a double or triple bond, and Z represents phenyl, fluorinated phenyl, biphenyl, cycloalkyl or halogenated alkyl group."

(2) On p.4 (p.4) of the description, formula 1 is amended as follows:



(3) On p.3, 1.12-14 (p.3, 1.10-12) of the description, "Therefore, it is ... cancer cell metastasis." is amended as follows.

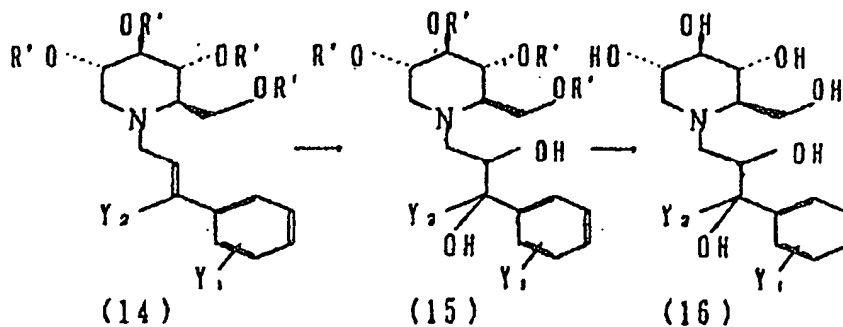
"Therefore, it is expected that suppression of cancer cell metastasis further improves the effectiveness of current cancer treatments."

(4) On p.15 in the 9th line from the bottom (p.12, 1.4-5) of the description, "... heating or leaving at room temperature with an aralkyl- or aralkenylation agent ..." is amended as follows.

"... heating or leaving at room temperature 1-

nojirimycin with an aralkyl- or aralkenylation agent ..."

(5) On p.16 (p.13) of the description, formulae (14), (15) and (16) are amended as follows.



(19)



JAPANESE PATENT OFFICE

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(21) Application number: **01158162**(22) Date of filing: **22.06.89**(71) Applicant: **TOSOH CORP**
(72) Inventor: **NAKANO KOICHI**
HASHIMOTO HIRONOBU
(54) **POLYHYDROXYPIPERIDINES AND PRODUCTION THEREOF**

removed by a catalytic reduction, thus obtaining the objective compound of formula I.

(57) Abstract:

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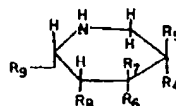
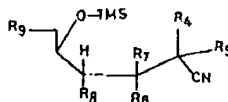
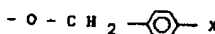
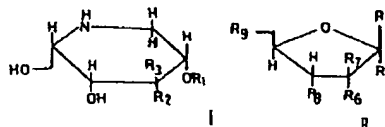
NEW MATERIAL: Compounds of formula I (R_1 is H or methyl; One of R_2 and R_3 is H and the other is OH).

EXAMPLE:

2-O-Benzyl-3,4,6-tri-O-acetyl-5-O-trimethylsilyl-D-allono nitrile.

USE: A glycosidase inhibitor.

PREPARATION: A ribofuranoside derivative of formula II [One of R_4 and R_5 is H and the other is alkoxy or formula III (X is R, CH_3 , OCH_3 or Cl); One of R_6 and R_7 is H and the other is acyloxy, etc.; R_8 is acyloxy, etc.; R_9 is acyloxy, azide, etc.] and an arabinofuranoside derivative are reacted with cyanotrimethylsilane in the presence of a Lewis acid and the resultant compound is then subjected to ring opening and carbon increase to obtain a compound of formula IV. The trimethylsilyl group of the resultant compound is substituted for a suitable elimination group and the cyano group thereof is subjected to ring closure by reduction to obtain a compound of formula V. Protective groups of the obtained compound of formula V are





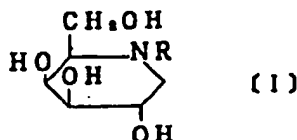
PCT

特許協力条約に基づいて公開された国際出願

(51) 国際特許分類5 C07D 211/46, A61K 31/445	AI	(11) 国際公開番号 WO 92/00277 (43) 国際公開日 1992年1月9日 (09.01.1992)
(21) 国際出願番号 POT/JP91/00866 (22) 国際出願日 1991年6月27日 (27. 06. 91) (30) 優先権データ 特願平 2/173629 1990年6月29日 (29. 06. 90) JP 特願平 3/36546 1991年2月4日 (04. 02. 91) JP (71) 出願人 (米国を除くすべての指定国について) 日本新薬株式会社 (NIPPON SHINYAKU CO., LTD.) [JP/JP] 〒601 京都府京都市南区吉祥院西ノ庄門口町14番地 Kyoto, (JP) (72) 発明者; および (75) 発明者/出願人 (米国についてのみ) 江連洋治 (EZURE, Yohji) [JP/JP] 〒520-21 滋賀県大津市野郷原2-21-22 Shiga, (JP) 丸尾重昭 (MARUO, Shigeaki) [JP/JP] 〒567 大阪府茨木市南安成2-2 13-104 Osaka, (JP) 宮崎京隆 (MIYAZAKI, Katsunori) [JP/JP] 〒020-91 岩手県盛岡市月が丘三丁目32-35 Iwate, (JP) 山田正典 (YAMADA, Naoyoshi) [JP/JP] 〒607 京都府京都市山科区大宅坂ノ辻町29-4-204 Kyoto, (JP) (74) 代理人 弁理士 片岡 宏, 外 (KATAOKA, Hiroshi et al.) 〒601 京都府京都市南区吉祥院西ノ庄門口町14番地 日本新薬株式会社内 Kyoto, (JP)	(81) 指定国 AT (欧州特許), BE (欧州特許), OA, OH (欧州特許), DE (欧州特許), DK (欧州特許), ES (欧州特許), FR (欧州特許), GB (欧州特許), GR (欧州特許), IT (欧州特許), JP, LU (欧州特許), NL (欧州特許), NO, SE (欧州特許), US. 添付公開書類 国際調査報告書	

(54) Title: PIPERIDINE DERIVATIVE

(54) 発明の名称 ピペリジン誘導体

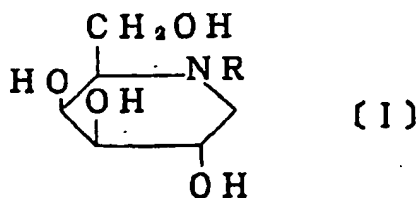


(57) Abstract

A 3,4,5-trihydroxypiperidine derivative of general formula (I), having a β -galactosidase inhibitory action and therefore usable as a carcinostatic agent, wherein R represents a C_1 to C_{18} saturated or unsaturated hydrocarbon group which may be substituted with a linear, branched or cyclic group.

(57) 要約

本発明に係る化合物は次の一般式〔I〕



(式中Rは、直鎖状、分枝状若しくは環状の置換基を有していてもよい炭素数1～18の飽和炭化水素又は不飽和炭化水素を示す。)で表される3, 4, 5、-トリヒドロキシピペリジン誘導体である。

本発明化合物はβ-ガラクトシダーゼ阻害作用を有しているので、制癌剤として使用しうるものである。

情報としての用途のみ

PCTに基づいて公開される国際出願のパンフレット第1頁にPCT加盟国を同定するために使用されるコード

AT オーストリア
AU オーストラリア
BB バルバドス
BE ベルギー
BF ブルキナ・ファソ
BG ブルガリア
BJ ベナン
BR ブラジル
CA カナダ
CF 中央アフリカ共和国
CG コンゴ
CH スイス
CI コート・ジボアール
CM カメルーン
CS チェコスロバキア
DE ドイツ
DK デンマーク

ES スペイン
FI フィンランド
FR フランス
GA ガボン
GI ギニア
GB イギリス
GR ギリシャ
HU ハンガリー
IT イタリア
JP 日本
KP 朝鮮民主主義人民共和国
KR 大韓民国
LI リヒテンシュタイン
LK スリランカ
LU ルクセンブルグ
MC モナコ
MG マダガスカル

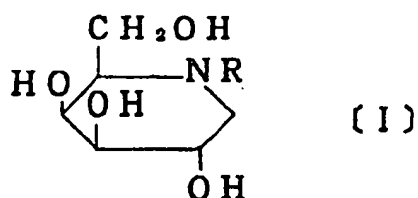
ML マリ
MN モンゴル
MR モーリタニア
MW マラウイ
NL オランダ
NO ノルウェー
PL ポーランド
RO ルーマニア
SD スーダン
SE スウェーデン
SN セネガル
SU ソビエト連邦
TD チャド
TG トーゴ
US 米国

明 細 書

ピペリジン誘導体

技 術 分 野

本発明は、次の一般式〔I〕で表されるピペリジン誘導体に関する。



式中Rは、直鎖状、分枝状若しくは環状の置換基を有していてもよい炭素数1～18の飽和炭化水素又は不飽和炭化水素を示す。

背 景 技 術

RNAウイルスで癌化した3T3線維芽細胞では、グルコシダーゼ活性、特にβ-ガラクトシダーゼ活性が上昇することがボスマンらにより報告されている(H. B. Bosmann et al., Biochem. Biophys. Acta, 264, 339(1972))。従って、β-ガラクトシダーゼ阻害物質は、制癌剤又は癌の転移抑制剤として利用できる可能性を有しており、これまで種々研究され、例えば、特願昭57-74090；特公昭53-31238；特願昭60-123135；The Journal of Antibiotics, 28, 1006(1975)；同32(3), 212(1979)；同32(3), 217(1979)等に記載されている。

発 明 の 開 示

β -ガラクトシダーゼ阻害物質を制癌剤として利用するためには、より強い阻害活性を有するもの程、投与量、副作用等の点で有利であることは容易に想像され得る。

そこで、本発明者らは、公知化合物よりもより阻害活性の強い化合物を見出すことを主目的として検討を行った。

本発明者らは、鋭意検討の結果、上記一般式〔I〕で表される3, 4, 5-トリヒドロキシピペリジン誘導体又はその薬理学的に許容される塩が強い阻害活性を有することを見出し、ようやく本発明を完成するに至った。本発明化合物は、文献未記載の新規化合物である。

ここに直鎖状、分枝状若しくは環状の飽和又は不飽和の炭化水素としては、メチル、エチル、*n*-プロピル、イソプロピル、ブチル、イソブチル、*sec*-ブチル、*tert*-ブチル、*n*-ヘプチル、*n*-ドデシル、ビニル、アリル、イソプロベニル、ブテニル、ヘブテニル、デセニル、エチニル、プロピニル、シクロプロピルメチル、シクロブチルメチル、シクロペンチルメチル、シクロヘキシルメチル、シクロプロベニルメチル、シクロブテニルメチル、シクロペンテニルメチル、シクロヘキセニルメチル、ベンジル等を挙げることができる。

置換基としては、メチル、エチル、*n*-プロピル、イソプロピル、*n*-ブチル、イソブチル、*sec*-ブチル、*tert*-ブチル、ヘプチル、水酸基、シアノ、フッ素、塩素、臭素、ヨウ素、ニトロ、ニトロソ、ホルミル、アセチル、プロピオニル、ヘキサノイル、ラウロイル、ベンゾイル、トルオイル、シンナモイル、メトキシ、エトキシ、プロポキシ、イソプロポキシ、

ブトキシ、ペンチルオキシ、フェノキシ、ベンジルオキシ、メチレンジオキシ、エチレンジオキシ、カルボキシ、メトキシカルボニル、エトキシカルボニル、プロポキシカルボニル、ホルミルオキシ、アセトキシ、ベンゾイルオキシ、アミノ、メチルアミノ、ジメチルアミノ、エチルアミノ、ジエチルアミノ、エチルメチルアミノ、アセチルアミノ、ベンゾイルアミノ、メトキシカルボニルアミノ、エトキシカルボニルアミノ、プロポキシカルボニルアミノ、アセチルメチルアミノ、スルホ、スルファモイル、スルホアミノ、カルバモイル、メチルカルバモイル、ジメチルカルバモイル、エチルカルバモイル、ジエチルカルバモイル、プロピルカルバモイル、ブチルカルバモイル、ウレイド、メチルウレイド、ジメチルウレイド、エチルウレイド、ジエチルウレイド、エチルメチルウレイド、フェニルウレイド、チオウレイド、メチルチオウレイド、ジメチルチオウレイド、エチルチオウレイド、ジエチルチオウレイド、エチルメチルチオウレイド、フェニルチオウレイド、グアニジノ等を、及び上記置換基の1種以上で置換されていてもよいフェニル、フェノキシ、トシル、シクロプロピル、シクロブチル、シクロペンチル、シクロヘキシル、シクロプロベニル、シクロブテニル、シクロペンテニル、シクロヘキセニル、ピロリル、チエニル、フリル、テオニル、ピリジル、ピペリジノ、ピペリジル、モルホルル、キノリル、インドリル、フタルイミド、アニリノ等を挙げることができる。更に、 $O-\beta-D$ -グルコピラノシル、 $S-\beta-D$ -グルコピラノシル等も置換基として挙げることができる。

また、薬理学的に許容される塩としては、リチウム塩、ナトリウム塩、カリウム塩、カルシウム塩等の金属塩の他、エタノールアミン塩、ジエタノールアミン塩等の有機塩基の塩、塩酸塩、硫酸塩、リン酸塩等の無機酸の塩、酢酸塩、メタンスルホン酸塩、コハク酸塩、乳酸塩、フマル酸塩、マレイン酸塩等の有機酸塩等を挙げることができる。

本発明化合物は、糖蛋白質糖鎖におけるガラクトースプロセッシングに係る酵素の阻害剤として利用できる可能性があり、糖蛋白質糖鎖プロセッシングの研究用試薬、糖鎖プロセッシングに係る α -グルコシダーゼ阻害剤、例えば、カスタノスペルミン、1-デオキシノジリマイシンが抗ウイルス作用 (Fleet et al., *PNAS* Lett., 237, 128(1988))、癌細胞転移抑制作用 (G. Pulverer et al., *J. Cancer. Res. Clin. Oncol.*, 114, 217(1988))又は免疫調節作用を有するように、それらの作用を持つ薬剤として期待される。

更に、食品、例えば、砂糖、ポテト、ジュース、ビール、チョコレート、ジャム又は飴等に本発明化合物の有効量を1種以上加えて保存性を高めることもできる。

本発明の要旨は、上記化合物〔I〕そのものにある。

本発明化合物の具体例としては、N-メチル-1-デオキシガラクトスタチン、N-エチル-1-デオキシガラクトスタチン、N-プロピル-1-デオキシガラクトスタチン、N-(2, 3-ジメチルブチル)-1-デオキシガラクトスタチン、N-(2, 2, 3-トリメチルペンチル)-1-デオキシガラクトスタチン、N-tert-ブチル-1-デオキシガ

ラクトスタチン、N-n-ペンチル-1-デオキシガラクトスタチン、N-イソペンチル-デオキシガラクトスタチン、N-sec-ペンチル-1-デオキシガラクトスタチン、N-(3-エチル-2-イソプロピルペンチル)-1-デオキシガラクトスタチン、N-ヘキシル-1-デオキシガラクトスタチン、N-ヘプチル-1-デオキシガラクトスタチン、N-イソヘキシル-1-デオキシガラクトスタチン、N-イソヘプチル-1-デオキシガラクトスタチン、N-オクチル-1-デオキシガラクトスタチン、N-イソオクチル-1-デオキシガラクトスタチン、N-デシル-1-デオキシガラクトスタチン、N-ドデシル-1-デオキシガラクトスタチン、N-テトラデシル-1-デオキシガラクトスタチン、N-ヘキサデシル-1-デオキシガラクトスタチン、N-オクタデシル-1-デオキシガラクトスタチン、N-シクロプロピルメチル-1-デオキシガラクトスタチン、N-シクロペンチルメチル-1-デオキシガラクトスタチン、N-シクロヘキシルメチル-1-デオキシガラクトスタチン、N-(2-ヒドロキシエチル)-1-デオキシガラクトスタチン、N-(3-ヒドロキシプロピル)-1-デオキシガラクトスタチン、N-(4-ヒドロキシブチル)-1-デオキシガラクトスタチン、N-(5-ヒドロキシペンチル)-1-デオキシガラクトスタチン、N-(2-ヒドロキシ-3-メチルブチル)-1-デオキシガラクトスタチン、N-(2,3-ジヒドロキシプロピル)-1-デオキシガラクトスタチン、N-(2-メトキシエチル)-1-デオキシガラクトスタチン、N-

(2-プロポキシエチル)-1-デオキシガラクトスタチン、
N-(2-アセトキシエチル)-1-デオキシガラクトスタチン、N-(4-ベンゾイルオキシブチル)-1-デオキシガラクトスタチン、N-(2-アミノエチル)-1-デオキシガラクトスタチン、N-(2-ジメチルアミノエチル)-1-デオキシガラクトスタチン、N-(2-アセチルアミノエチル)-1-デオキシガラクトスタチン、N-(2-ベンゾイルアミノエチル)-1-デオキシガラクトスタチン、N-(2-プロポキシカルボニルアミノエチル)-1-デオキシガラクトスタチン、N-(2-(N', N'-アセチルメチル)アミノエチル)-1-デオキシガラクトスタチン、N-(2-(N'-メチルウレイド)エチル)-1-デオキシガラクトスタチン、N-(2-(N'-フェニルウレイド)エチル)-1-デオキシガラクトスタチン、N-(2-(N'-メチルチオウレイド)エチル)-1-デオキシガラクトスタチン、N-(2-(N'-フェニルチオウレイド)エチル)-1-デオキシガラクトスタチン、N-(3-アミノプロピル)-1-デオキシガラクトスタチン、N-(3-アセチルアミノプロピル)-1-デオキシガラクトスタチン、N-(3-ベンゾイルアミノプロピル)-1-デオキシガラクトスタチン、N-(3-(N'-メチルウレイド)プロピル)-1-デオキシガラクトスタチン、N-シンナミル-1-デオキシガラクトスタチン、2-フェノキシエチル-1-デオキシガラクトスタチン、N-(p-エトキシカルボニルフェノキシ)エチル)-1-デオキシガラクトスタチン、N-(2-ベンジル

オキシエチル) - 1-デオキシガラクトスタチン、N- (3-フェノキシカルボニルプロピル) - 1-デオキシガラクトスタチン、N- (4-アミノブチル) - 1-デオキシガラクトスタチン、N-アリル-1-デオキシガラクトスタチン、N- (2-ブテニル) - 1-デオキシガラクトスタチン、N- (3-ブテニル) - 1-デオキシガラクトスタチン、N- (5-ヘキセニル) - 1-デオキシガラクトスタチン、N- (9-デセニル) - 1-デオキシガラクトスタチン、N-カルボキシメチル-1-デオキシガラクトスタチン、N- (2-カルボキシエチル) - 1-デオキシガラクトスタチン、N-エトキシカルボニルエチル-1-デオキシガラクトスタチン、N-カルバモイルメチル-1-デオキシガラクトスタチン、N- (N'-エチルカルバモイルメチル) - 1-デオキシガラクトスタチン、N- (N'-ブチルカルバモイルメチル) - 1-デオキシガラクトスタチン、N- (3-スルホプロピル) - 1-デオキシガラクトスタチン、N- (3-スルファモイルプロピル) - 1-デオキシガラクトスタチン、N- (o-カルボキシベンジル) - 1-デオキシガラクトスタチン、N- (o-ニトロベンジル) - 1-デオキシガラクトスタチン、N- (5-ブロモ-2-ヒドロキシベンジル) - 1-デオキシガラクトスタチン、N-ベンゾイルメチル-1-デオキシガラクトスタチン、N- (4-ヒドロキシ-3-メトキシベンジル) - 1-デオキシガラクトスタチン、N- (2-プロピニル) - 1-デオキシガラクトスタチン、N- (p-ヒドロキシベンジル) - 1-デオキシガラクトスタチン、N-

— (4-ヒドロキシ-3-メトキシ-5-ニトロベンジル)
— 1-デオキシガラクトスタチン、N- (4-ニトロ-2-スルホベンジル) — 1-デオキシガラクトスタチン、N- (2-ヒドロキシ-4, 6-ジメトキシベンジル) — 1-デオキシガラクトスタチン、N- (2-メチルチオベンジル) — 1-デオキシガラクトスタチン、ジソジウム N- (2, 4-ジスルホネートベンジル) — 1-デオキシガラクトスタチン、N- (2-クロロ-5-ニトロベンジル) — 1-デオキシガラクトスタチン、N- (2-クロロ-6-ニトロベンジル) — 1-デオキシガラクトスタチン、N- (4-クロロ-3-ニトロベンジル) — 1-デオキシガラクトスタチン、N- (5-クロロ-2-ニトロベンジル) — 1-デオキシガラクトスタチン、N- (o-ブromoベンジル) — 1-デオキシガラクトスタチン、N- (p-ブromoベンジル) — 1-デオキシガラクトスタチン、N- (o-クロロベンジル) — 1-デオキシガラクトスタチン、N- (m-クロロベンジル) — 1-デオキシガラクトスタチン、N- (p-クロロベンジル) — 1-デオキシガラクトスタチン、N- (o-フルオロベンジル) — 1-デオキシガラクトスタチン、N- (m-フルオロベンジル) — 1-デオキシガラクトスタチン、N- (p-フルオロベンジル) — 1-デオキシガラクトスタチン、N- (o-ニトロベンジル) — 1-デオキシガラクトスタチン、N- (4-ヒドロキシ-3-ニトロベンジル) — 1-デオキシガラクトスタチン、N- (5-ヒドロキシ-2-ニトロベンジル) — 1-デオキシガラクトスタチン、N- (m-ヒド

ロキシベンジル) - 1-デオキシガラクトスタチン、N-(
p-ヒドロキシベンジル) - 1-デオキシガラクトスタチン、
N-(o-ヒドロキシベンジル) - 1-デオキシガラクトス
タチン、N-(2, 5-ジヒドロキシベンジル) - 1-デオ
キシガラクトスタチン、N-(3, 4-ジヒドロキシベンジ
ル) - 1-デオキシガラクトスタチン、N-(p-カルボキ
シベンジル) - 1-デオキシガラクトスタチン、N-(3,
4-メチレンジオキシベンジル) - 1-デオキシガラクトス
タチン、N-(3-カルボキシ-4-ヒドロキシベンジル)
- 1-デオキシガラクトスタチン、N-(o-メチルベンジ
ル) - 1-デオキシガラクトスタチン、N-(p-メチルベ
ンジル) - 1-デオキシガラクトスタチン、N-(o-メト
キシベンジル) - 1-デオキシガラクトスタチン、N-(m
-メトキシベンジル) - 1-デオキシガラクトスタチン、N
-(4-ヒドロキシ-3-メトキシベンジル) - 1-デオキ
シガラクトスタチン、N-(3-ヒドロキシ-4-メトキシ
ベンジル) - 1-デオキシガラクトスタチン、N-(3, 4
-ジメトキシベンジル) - 1-デオキシガラクトスタチン、
N-(p-アセチルアミノベンジル) - 1-デオキシガラク
トスタチン、N-(2, 5-ジメチルベンジル) - 1-デオ
キシガラクトスタチン、N-(o-エトキシベンジル) - 1
-デオキシガラクトスタチン、N-(2-メチル-4-メト
キシベンジル) - 1-デオキシガラクトスタチン、N-(3,
5-ジメトキシベンジル) - 1-デオキシガラクトスタチン、
N-(p-ジメチルアミノベンジル) - 1-デオキシガラク

トスタチン、N- (3, 4, 5-トリメトキシベンジル) -
1-デオキシガラクトスタチン、N- (2, 4, 5-トリメ
トキシベンジル) -1-デオキシガラクトスタチン、N- (
2, 3-エポキシプロピル) -1-デオキシガラクトスタチ
ン、N- (3-フタルイミドプロピル) -1-デオキシガラ
クトスタチン、N- (2-フタルイミドエチル) -1-デオ
キシガラクトスタチン、N- (2-ピリジル) メチル-1-
デオキシガラクトスタチン、N- (2- (S- β -D-グル
コピラノシル-2-メルカプト) エチル) -1-デオキシガ
ラクトスタチン、N- (2- (O- β -D-グルコピラノシ
ル) エチル) -1-デオキシガラクトスタチン、N- (2-
フリル) メチル-1-デオキシガラクトスタチン、N- (3-
インドリル) メチル-1-デオキシガラクトスタチン、N-
- (2- (5-プロモチエニル)) メチル-1-デオキシガ
ラクトスタチン、N- (2-ピロリル) メチル-1-デオキ
シガラクトスタチン、N- (3-ピリジル) メチル-1-デ
オキシガラクトスタチン、N- (4-ピリジル) メチル-1-
デオキシガラクトスタチン、N-ベンジル-1-デオキシ
ガラクトスタチン等を挙げることができる。

本発明化合物を医薬として投与する場合、本発明化合物は
そのまま又は医薬的に許容される無毒性かつ不活性の担体中
に、例えば 0.1%~99.5%、好ましくは 0.5%~90%含有す
る医薬組成物として、人を含む動物に投与される。

担体としては、固形、半固形、又は液状の希釈剤、充填剤、
及びその他の処方用の助剤一種以上が用いられる。医薬組成

物は、投与単位形態で投与することが望ましい。本発明医薬組成物は、経口投与、組織内投与、局所投与（経皮投与等）又は経直腸的に投与することができる。これらの投与方法に適した剤型で投与されるのはもちろんである。例えば、経口投与が特に好ましい。

β -ガラクトシダーゼ阻害剤としての用量は、年齢、体重等の患者の状態、投与経路、病気の性質と程度等を考慮した上で設定することが望ましいが、通常は、成人に対して本発明の有効成分量として、1日あたり、0.1 mg～3 g/日/ヒトの範囲が、好ましくは、1 mg～100 mg/日/ヒトの範囲が一般的である。場合によっては、これ以下でも足りるし、また逆にこれ以上の用量を必要とすることもある。また、1日1～3回に分割して投与することが望ましい。

経口投与は固形又は液状の用量単位、例えば、末剤、散剤、錠剤、糖衣剤、カプセル剤、顆粒剤、懸濁剤、液剤、シロップ剤、ドロップ剤、舌下錠その他の剤型によって行うことができる。

末剤は活性物質を適当な細かさにより製造される。散剤は活性物質を適当な細かさとし、ついで同様に細かくした医薬用担体、例えば澱粉、マンニトールのような可食性炭水化物その他と混合することにより製造される。必要に応じ風味剤、保存剤、分散剤、着色剤、香料その他のものを混じてもよい。

カプセル剤は、まず上述のようにして粉末状となった末剤や散剤あるいは錠剤の項で述べるように顆粒化したものを、

例えばゼラチンカプセルのようなカプセル外皮の中へ充填することにより製造される。滑沢剤や流動化剤、例えばコロイド状のシリカ、タルク、ステアリン酸マグネシウム、ステアリン酸カルシウム、固形のポリエチレングリコールのようなものを粉末状態のものに混合し、然るのちに充填操作を行うこともできる。崩壊剤や可溶化剤、例えばカルボキシメチルセルロース、カルボキシメチルセルロースカルシウム、低置換度ヒドロキシプロピルセルロース、クロスカルメロースナトリウム、カルボキシスターチナトリウム、炭酸カルシウム、炭酸ナトリウム、を添加すれば、カプセル剤が摂取されたときの医薬の有効性を改善することができる。

また、本品の微粉末を植物油、ポリエチレングリコール、グリセリン、界面活性剤中に懸濁分散し、これをゼラチンシートで包んで軟カプセル剤とすることができる。錠剤は粉末混合物を作り、顆粒化もしくはスラグ化し、ついで崩壊剤又は滑沢剤を加えたのち打錠することにより製造される。粉末混合物は、適当に粉末化された物質を上述の希釈剤やベースと混合し、必要に応じ結合剤（例えば、カルボキシメチルセルロースナトリウム、ヒドロキシプロピルセルロース、メチルセルロース、ヒドロキシプロピルメチルセルロース、ゼラチン、ポリビニルピロリドン、ポリビニルアルコールなど）、溶解遅延化剤（例えば、パラフィン、ワックス、硬化ヒマシ油など）、再吸収剤（例えば、四級塩）や吸着剤（例えばベントナイト、カオリン、リン酸ジカルシウムなど）をも併用してもよい。粉末混合物は、まず結合剤、例えばシロップ、

澱粉糊、アラビアゴム、セルロース溶液又は高分子物質溶液で湿らせ、ついで篩を強制通過させて顆粒とすることができる。このように粉末を顆粒化するかわりに、まず打錠機にかけたのち、得られる不完全な形態のスラグを破砕して顆粒にすることも可能である。

このようにして作られる顆粒は、滑沢剤としてステアリン酸、ステアリン酸塩、タルク、ミネラルオイルその他を添加することにより、互いに付着することを防ぐことができる。このように滑沢化された混合物をついで打錠する。

また薬物は、上述のように顆粒化やスラグ化の工程を経ることなく、流動性の不活性担体と混合したのちに直接打錠してもよい。シェラックの密閉被膜からなる透明又は半透明の保護被覆、糖や高分子材料の被覆、及び、ワックスよりなる磨上被覆の如きも用いる。

他の経口投与剤型、例えば溶液、シロップ、エリキシルなどもまたその一定量が薬物の一定量を含むように用量単位形態にすることができる。シロップは、化合物を適当な香味水溶液に溶解して製造され、またエリキシルは非毒性のアルコール性担体を用いることにより製造される。懸濁剤は、化合物を非毒性担体中に分散させることにより処方される。可溶化剤や乳化剤（例えば、エトキシ化されたイソステアリルアルコール類、ポリオキシエチレンソルビトールエステル類）、保存剤、風味賦与剤（例えば、ペパメント油、サッカリン）その他もまた必要に応じ添加することができる。

必要とあらば、経口投与のための用量単位処方はいくつ

カプセル化してもよい。該処方はまだ被覆をしたり、高分子・ワックス等中にうめこんだりすることにより作用時間の延長や持続放出をもたらすこともできる。

組織内投与は、皮下・筋肉又は静脈内注射用としたところの液状用量単位形態、例えば溶液や懸濁剤の形態を用いることによって行うことができる。これらのものは、化合物の一定量を、注射の目的に適合する非毒性の液状担体、例えば水性や油性の媒体に懸濁し又は溶解し、ついで該懸濁液又は溶液を滅菌することにより製造される。又は、化合物の一定量をバイアルにとり、そのうち該バイアルとその内容物を滅菌し密閉してもよい。投与直前に溶解又は混合するために、粉末又は凍結乾燥した有効成分に添えて、予備的のバイアルや担体を準備してもよい。注射液を等張にするために非毒性の塩や塩溶液を添加してもよい。さらに安定剤、保存剤、乳化剤のようなものを併用することもできる。

直腸投与は、化合物を低融点の水に可溶又は不溶の固体、例えばポリエチレングリコール、カカオ脂、半合成の油脂（例えば、ウイテプゾール、登録商標）、高級エステル類（例えばバルミチン酸ミリスチルエステル）及びそれらの混合物に溶解又は懸濁させて製造した坐剤を用いることによって行うことができる。

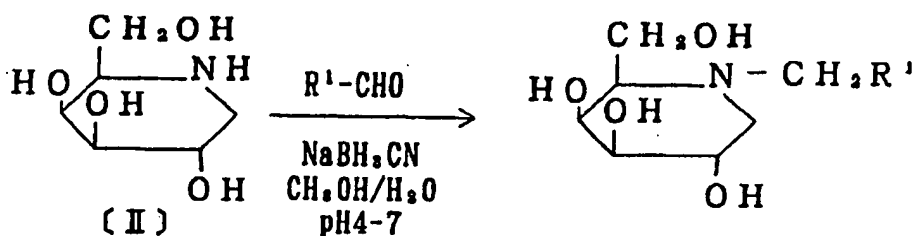
（合成例）

本発明化合物は、1-デオキシガラクトスタチン〔II〕の窒素に結合している水素を公知の方法、例えばカルボニル化合物及び水素供与還元剤により還元的に置換する方法若しく

は直接前記種々の置換基を有する試薬で置換する方法又は〔Ⅱ〕の窒素を公知の方法によりアシル化し還元する方法等によって合成することができる。具体的には、以下の方法を挙げることができる。

還元的置換

次の反応式に従って、本発明化合物は、化合物〔Ⅱ〕を適当な溶媒、例えば、水／アルコール混合物中において、ケトン又はアルデヒド及び適当な還元剤、例えば、水素化シアノホウ素アルカリ金属、ジアルキルアミノボラン、水素化ホウ素アルカリ金属等、具体的には水素化シアノホウ素ナトリウム (NaBH_3CN)、水素化ホウ素ナトリウム／トリフルオロ酢酸又はラネーニッケル／水素等と 0～100℃ で反応させて得ることができる。



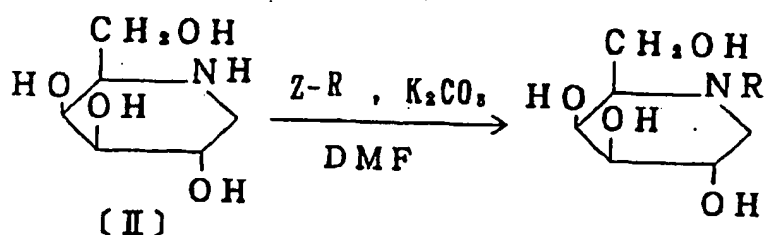
(上記 R^1 は、水素、水酸基又は前記 R に対すると同じ意味を示す。)

また、ロイカートーバラッハ (Leuekart-Wallach) 反応によることもできる。

直接置換

直接窒素に結合している水素を置換基に置き換えることによる本発明化合物の合成は、次の反応式に従って、化合物〔

Ⅱ〕を適当な溶媒、例えば、N,N-ジメチルホルムアミド（以下「DMF」という。）中でアルキル化剤（Z-R）及び適当な塩基、例えば、炭酸カリウムと0～100℃で反応させることによって行う。

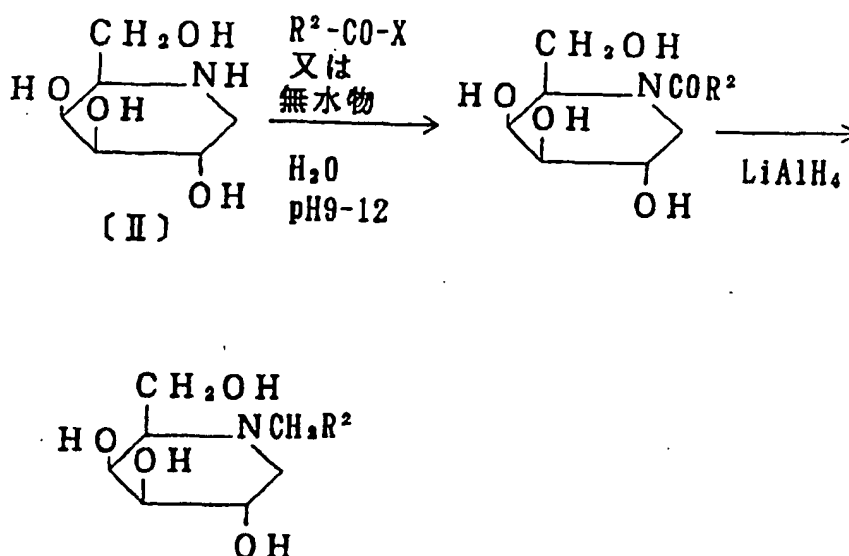


（上記 R は、前記と同じ。Z は、容易に離脱し且つアルキル化剤における通常の基、例えば、塩素、臭素又は沃素等のハロゲンを示す。）

アシル化合物の還元

アシル化合物の還元による本発明化合物の合成は、次の反応式に従って、化合物〔Ⅱ〕を適当な溶媒、例えば、水、水／アルコール混合物又は DMF 中にてアシルハライド（ $\text{R}^2\text{-CO-X}$ ）又は対応する無水物（ $\text{R}^2\text{-CO-O-CO-R}^2$ ）でアシル化し、適当な還元剤、例えば、水素化アルミニウムリチウム（ LiAlH_4 ）で還元することによって行う。

（以下次頁）



(上記 R^2 は、前記 R に対すると同じ意味を示す。 X は、ハロゲン、例えば、塩素、臭素又は沃素を示す。)

発明を実施するための最良の形態

以下、実施例、参考例及び試験例により本発明化合物を更に詳しく説明するが、言うまでもなく本発明は、これらに限定されない。

参考例 1 N-ベンジルオキシカルボニルモラノリンの合成

モラノリン 16.3g (0.1モル) 及び炭酸水素ナトリウム 8.4g (0.1モル) を水 160ml 及びクロロホルム 160ml に溶解し、氷冷下ベンジルオキシカルボニルクロライド 20.47g (0.12モル) を添加して激しく攪拌した。8時間後、再び氷冷下炭酸水素ナトリウム 0.84g (0.01モル) ベンジルオキシカルボニルクロライド 5.12g (0.03モル) を添加し、6時間反応した。そして、水層の pH を 5-6 に調整し、等量のクロロホルムで 3 回抽出を行なった後、水層を乾燥した。次いで、得られた

固形物に酢酸エチル及びエタノール（１：１）を加え不溶物を濾過し、濾液を乾燥して、オイル状の本化合物 30g（定量的）を得た。

参考例 2 N-ベンジルオキシカルボニル-4, 6-O-ベンジリデンモラノリンの合成

参考例 1 の化合物 29g (97ミリモル)、無水トルエンスルホン酸 3.34g (19ミリモル)、ベンズアルデヒドジメチルアセタール 29.4g (194ミリモル) 及び活性硫酸カルシウム 29g を DMF 290ml に加え、30℃にて24時間攪拌した。そして、反応液に強塩基性イオン交換樹脂（ダイヤイオン SA-11A OH⁻ 型）を加えて中和し、不溶物を濾過後、濾液を乾燥し、酢酸エチル及びヘキサンで結晶化して非晶質物質の本化合物 29g（収率72%）を得た。融点 134～138℃。

参考例 3 N-ベンジルオキシカルボニル-2, 3-ジ-O-ベンジル-4, 6-O-ベンジリデンモラノリンの合成

参考例 2 の化合物 27g (70ミリモル) 及び60%水素化ナトリウム 21g (875ミリモル) を DMF 1000ml に加え、30分間攪拌した。次いで氷冷下ベンジルブロマイド 170g (995ミリモル) を滴下し、二日間放置した。放置後、メタノールを加え、反応液を乾燥して得られたオイル状物質をクロロホルムに溶解し、水で数回抽出し、クロロホルム層を乾燥した。そして、得られたオイル状物質をシリカゲルカラムクロマト（ワコゲル C-200）に付し、ヘキサン-酢酸エチル（9：1）で溶出し、オイル状の本化合物 37.5g（収率94%）を得た。

参考例 4 N-ベンジルオキシカルボニル-2, 3-ジ-O

ーベンジルモラノリンの合成

参考例 3 の化合物 36.5g (64ミリモル) を酢酸 320ml 及び水 80ml に溶解し、60℃にて 6 時間攪拌した。攪拌後、反応液を乾燥し、本化合物を含有するオイル状物質 36.3g を得た。

参考例 5 N, 6-〇-カルバモイル-2, 3-ジ-〇-ベンジルモラノリンの合成

参考例 4 の化合物を含むオイル状物質 36.3g 及び炭酸カリウム 40g をメタノール 360ml 及び水 40ml に溶解し、60℃で 4 時間攪拌した。攪拌後、反応液を乾燥し、得られた固形物をクロロホルムに溶解して水で数回抽出した。そして、クロロホルム層を乾燥し、酢酸エチル及びヘキサンにて結晶化して非晶質物質 19g を得た。融点 110~111℃。

参考例 6 N, 6-〇-カルバモイル-2, 3-ジ-〇-ベンジル-4-〇-メシルモラノリンの合成

参考例 5 の化合物 19g (51ミリモル) 及びトリエチルアミン 15.6g (154ミリモル) をアセトン 200ml に溶解し、氷冷下メシルクロライド 9.8g (85ミリモル) を滴下し、30 分間攪拌した。攪拌後、反応液を乾燥し、0.1N 塩酸及び酢酸エチルで分配して酢酸エチル層を乾燥した。次いで、0.1N 炭酸水素ナトリウム及びクロロホルムで分配してクロロホルム層を乾燥した。そして、酢酸エチル、クロロホルム及びヘキサンにて結晶化し、本化合物 22.6g (収率 98%) を得た。融点 197~199℃。

参考例 7 N, 6-〇-カルバモイル-2, 3-ジ-〇-ベンジル-4-〇-ベンゾイル-1-デオキシガラクトスタチ

ンの合成

参考例 6 の化合物 22.1g (49ミリモル) 及び安息香酸リチウム 7.54g (58ミリモル) を DMF 30ml に溶解し、100℃で 2 日間攪拌した。攪拌後、反応液を乾燥し、0.2N 炭酸水素ナトリウム及び酢酸エチルで分配し、そして酢酸エチル層を乾燥し、オイル状の本化合物 24.5g (定量的) を得た。次いで、ジエチルエーテルにて非晶質化した。融点 135~137℃。

参考例 8 N, 6-O-カルバモイル-2, 3-ジ-O-ベンジルー1-デオキシガラクトスタチンの合成

参考例 7 の化合物 24g (50ミリモル) を塩化メチレン 450 ml 及びメタノール 100ml に溶解し、10N 水酸化ナトリウム 5 ml を添加し 50℃で 3 時間攪拌した。攪拌後、反応液に濃塩酸を加えて中和し、乾燥してクロロホルム及び水で分配した。そして、クロロホルム層を乾燥し、本化合物を含有するオイル状物質 20.9g を得た。

参考例 9 2, 3-ジ-O-ベンジルー1-デオキシガラクトスタチンの合成

参考例 8 の化合物を含むオイル状物質 20g 及び水酸化バリウム 8 水和物をメタノール 320ml 及び水 80ml に溶解し、還流下 4 時間攪拌した。攪拌後、反応液にドライアイス投入して 8500rpm で遠心分離を行ない沈澱物をメタノールで洗い再び遠心分離した。そして、上清を合わせて乾燥し、0.1N 塩酸及びクロロホルムで分配し、塩酸層を炭酸ナトリウムで弱塩基性にして本化合物を酢酸エチルで抽出した。次いで、酢酸エチル層を乾燥し、酢酸エチル及びヘキサンにて板状晶 11.82g

を得た。融点 126～128℃。

参考例10 1-デオキシガラクトスタチン塩酸塩の合成

参考例9の化合物 10gを液体アンモニアに溶解し、金属ナトリウム2.8gを加えてアセトンドライアイス中で30分間攪拌した。そして、反応液の青色が消えるまで塩化アンモニウムを加え、次いでアンモニアを気化させ残った固形物を強酸イオン交換樹脂（ダウエックス 50WX-2 H⁺型）のカラムに導入し、水洗後、1Nアンモニアで溶出し溶離液を乾燥した。続いて、強塩基性イオン交換樹脂（ダイヤイオンSA-11A OH⁻型）のカラムに導入し、水通過液を乾燥した。最後に得られたオイル状物質をエタノールに溶解し、濃塩酸で弱酸性に調整して本化合物 5.26g（収率90%）の結晶を得た。融点 237～239℃。

$[\alpha]_D$ 54.96 (20℃、C=0.997, H₂O)

元素分析値 (C₈H₁₄ClNO₄として)

計算値 (%) C: 36.10 H: 7.07 N: 7.02

実測値 (%) C: 35.97 H: 7.09 N: 7.04

実施例1 N-メチルー1-デオキシガラクトスタチンの合成

1-デオキシガラクトスタチン塩酸塩0.5g（2.5ミリモル）、35%ホルマリン溶液 0.64g（7.5ミリモル）及び水素化シアノホウ素ナトリウム 0.16g（2.5ミリモル）をメタノール12.5ml及び水 2.5mlに溶解し、pH値を氷酢酸によって4～5にし、この混合物を室温で2時間攪拌した。攪拌後、この溶液を強酸イオン交換樹脂（ダウエックス 50WX-2 H⁺型）

に導入し、イオン交換樹脂をメタノールで洗浄し、生成物をメタノール／濃アンモニア＝10：1で溶離した。溶離剤を回転蒸発機で蒸発乾固させた後、生成物を強塩基性イオン交換樹脂（ダイヤイオンSA-11A OH⁻型）に導入し、水通過液を蒸発乾固して得られた生成物をエタノールで再結晶して本発明化合物 0.34gを得た。融点 164～166℃。

$[\alpha]_D$ -3.27 (20℃、C=1.037, H₂O)

元素分析値 (C₇H₁₃NO₄として)

計算値 (%) C:47.45 H:8.53 N:7.90

実測値 (%) C:47.44 H:8.51 N:7.94

実施例 2 N-エチルー1-デオキシガラクトスタチンの合成

ホルマリンの代わりにアセトアルデヒドを用いて実施例 1と同様にして合成した。融点 159～161℃。

$[\alpha]_D$ -21.31 (20℃、C=1.032, H₂O)

元素分析値 (C₈H₁₇NO₄として)

計算値 (%) C:50.25 H:8.96 N:7.32

実測値 (%) C:49.96 H:8.85 N:7.31

実施例 3 N-n-プロピルー1-デオキシガラクトスタチンの合成

ホルマリンの代わりにプロピオンアルデヒドを用いて実施例 1と同様にして合成した。融点 120～122℃。

$[\alpha]_D$ -27.00 (20℃、C=0.985, H₂O)

元素分析値 (C₉H₁₉NO₄として)

計算値 (%) C:52.67 H:9.33 N:6.82

実測値 (%) C:52.52 H:9.21 N:6.87

実施例 4 N-イソブチル-1-デオキシガラクトスタチン
の合成

ホルマリンの代わりにイソブタナールを用いて実施例 1 と同様に合成し、塩酸塩として結晶化した。融点 142~145℃。

$[\alpha]_D$ 1.93 (20℃、C=0.516, H₂O)

元素分析値 (C₁₀H₂₁NO₄ · HCl として)

計算値 (%) C:46.97 H:8.67 N:5.48

実測値 (%) C:46.81 H:8.67 N:5.46

実施例 5 N-n-ヘプチル-1-デオキシガラクトスタチン
の合成

ホルマリンの代わりにヘプタナールを用いて実施例 1 と同様に合成した。融点 125~127℃。

$[\alpha]_D$ -25.92 (20℃、C=1.003, MeOH)

元素分析値 (C₁₃H₂₇NO₄ として)

計算値 (%) C:59.74 H:10.41 N:5.36

実測値 (%) C:59.25 H:10.41 N:5.37

実施例 6 N-n-ドデシル-1-デオキシガラクトスタチン
の合成

ホルマリンの代わりにドデカナールを用いて実施例 1 と同様に合成した。融点 124~128℃。

$[\alpha]_D$ -14.41 (20℃、C=0.999, DMSO)

元素分析値 (C₁₆H₃₇NO₄ として)

計算値 (%) C:65.22 H:11.25 N:4.23

実測値 (%) C:64.79 H:10.91 N:4.22

実施例 7 N-(3-フェニルプロピル)-1-デオキシガラクトスタチンの合成

ホルマリンの代わりに3-フェニルプロパナールを用いて実施例1と同様にして合成した。融点 100~104℃。

$[\alpha]_D$ -25.94 (20℃、C=0.501, MeOH)

元素分析値 (C₁₅H₂₃NO₄ として)

計算値 (%) C:64.04 H:8.24 N:4.98

実測値 (%) C:64.01 H:8.35 N:5.02

実施例 8 N-p-クロロベンジル-1-デオキシガラクトスタチンの合成

ホルマリンの代わりにp-クロロベンズアルデヒドを用いて実施例1と同様にして合成し、塩酸塩として結晶化した。融点 101~104℃。

$[\alpha]_D$ 12.59 (20℃、C=1.000, H₂O)

元素分析値 (C₁₅H₂₃NO₄·HCl·1/2H₂O として)

計算値 (%) C:46.86 H:6.05 N:4.20

実測値 (%) C:46.75 H:6.22 N:4.13

実施例 9 N-アリル-1-デオキシガラクトスタチンの合成

1-デオキシガラクトスタチン塩酸塩0.3g (1.5 ミリモル)、無水炭酸カリウム 0.21g (1.7 ミリモル) をDMF 5 ml に懸濁し、氷冷中、アリルブロマイド0.2g (1.65ミリモル) を加え、この混合物を室温で12時間攪拌した。攪拌後、塩を濾別し、混合物を実施例1と同様に樹脂処理を行ない本発明

化合物 0.19gを得た。融点 144～146℃。

$[\alpha]_D$ -14.69 (20℃、C=1.007, H₂O)

元素分析値 (C₉H₁₇NO₄として)

計算値 (%) C:53.19 H:8.43 N:6.89

実測値 (%) C:53.00 H:8.49 N:6.79

実施例10 N-シンナミル-1-デオキシガラクトスタチン
の合成

アリルブロマイドの代わりにシンナミルブロマイドを用いて実施例9と同様にして合成した。融点 66～69℃。

$[\alpha]_D$ -40.63 (20℃、C=0.507, MeOH)

元素分析値 (C₁₅H₂₁NO₄・1/2H₂Oとして)

計算値 (%) C:62.48 H:7.69 N:4.86

実測値 (%) C:62.56 H:7.68 N:4.89

実施例11 N-メトキシエチル-1-デオキシガラクトスタチン
の合成

アセトアルデヒドを用いて実施例1と同様にして合成した。融点 110～111℃。

$[\alpha]_D$ -14.42 (20℃、C=1.040, H₂O)

FAB-MAS m/z 222 (M + +1)

元素分析値 (C₉H₁₉NO₅として)

計算値 (%) C:48.86 H:8.66 N:6.33

実測値 (%) C:48.43 H:8.83 N:6.37

実施例12 N-(p-フェニルベンジル)-1-デオキシガラクトスタチン
の合成

フェニルベンズアルデヒドを用いて実施例1と同様にして

合成した。融点 177～178℃。

$[\alpha]_D$ -24.03 (20℃、C=0.491, DMSO)

FAB-MAS m/z 330 ($M + +1$)

元素分析値 ($C_{10}H_{13}NO_4 \cdot 1/4H_2O$ として)

計算値 (%) C:68.35 H:7.09 N:4.19

実測値 (%) C:68.71 H:7.35 N:4.20

実施例13 N-n-ペンチルニルー1-デオキシガラクトスタチンの合成

ホルマリンの代わりにn-ペンタナールを用いて実施例1と同様にして合成した。融点 115～116℃。

$[\alpha]_D$ -26.30 (20℃、C=0.517, H_2O)

FAB-MAS m/z 234 ($M + +1$)

元素分析値 ($C_{11}H_{15}NO_4$ として)

計算値 (%) C:56.63 H:9.94 N:6.00

実測値 (%) C:56.35 H:9.63 N:6.06

実施例14 N-p-メトキシベンジルー1-デオキシガラクトスタチンの合成

ホルマリンの代わりにp-アニスアルデヒドを用いて実施例1と同様にして合成した。融点 122～124℃。

$[\alpha]_D$ -26.62 (20℃、C=0.586, H_2O)

FAB-MAS m/z 284 ($M + +1$)

元素分析値 ($C_{14}H_{17}NO_4$ として)

計算値 (%) C:59.35 H:7.47 N:4.94

実測値 (%) C:59.05 H:7.43 N:4.91

実施例15 N-(p-メチルチオベンジル)-1-デオキシ

ガラクトスタチンの合成

ホルマリンの代わりに p-メチルチオベンズアルデヒドを用いて実施例 1 と同様にして合成した。融点 120～123℃。

PAB-MAS m/z 300 (M + +1)

元素分析値 (C₁₄H₂₁NO₄S · 1/2 H₂O として)

計算値 (%) C:54.52 H:7.20 N:4.54

実測値 (%) C:54.38 H:6.91 N:4.72

実施例16 N-フェニルエチル-1-デオキシガラクトスタチンの合成

ホルマリンの代わりにフェニルアセトアルデヒドを用いて実施例 1 と同様にして合成した。融点 188～190℃。

PAB-MAS m/z 268 (M + +1)

元素分析値 (C₁₄H₂₁NO₄ として)

計算値 (%) C:62.90 H:7.93 N:5.24

実測値 (%) C:62.57 H:8.01 N:5.32

実施例17 N-(1'-デオキシガラクトチトル)-1-デオキシガラクトスタチンの合成

ホルマリンの代わりに D-ガラクトースを用いて実施例 1 と同様にして合成した。融点 79～82℃。

[α]_D -17.60 (20℃、C=0.284, H₂O)

PAB-MAS m/z 328 (M + +1)

元素分析値 (C₁₂H₂₅NO₉ · 2H₂O として)

計算値 (%) C:39.67 H:8.04 N:3.85

実測値 (%) C:39.70 H:8.32 N:4.15

実施例18 N-(p-アセトアミドベンジル)-1-デオキ

シガラクトスタチンの合成

ホルマリンの代わりにp-アセトアミノベンズアルデヒドを用いて実施例1と同様にして合成した。融点 95~96℃。

$[\alpha]_D$ -20.73 (20℃、C=0.492, H₂O)

FAB-MAS m/z 311 (M + +1)

元素分析値 (C₁₅H₂₂N₂O₅ · 3/4H₂Oとして)

計算値 (%) C:55.63 H:7.31 N:8.65

実測値 (%) C:55.89 H:7.55 N:8.64

実施例19 N-フェニルプロピル-1-デオキシガラクトスタチンの合成

ホルマリンの代わりにフェニルプロパナールを用いて実施例1と同様にして合成した。融点 100~104℃。

$[\alpha]_D$ -25.94 (20℃、C=0.501, MeOH)

FAB-MAS m/z 282 (M + +1)

元素分析値 (C₁₅H₂₃NO₄として)

計算値 (%) C:64.04 H:8.24 N:4.98

実測値 (%) C:64.01 H:8.35 N:5.02

実施例20 N-シクロヘキシルメチル-1-デオキシガラクトスタチンの合成

ホルマリンの代わりにシクロヘキサンカルボキサルデヒドを用いて実施例1と同様にして合成した。融点 72~73℃。

$[\alpha]_D$ -39.72 (20℃、C=0.715, H₂O)

FAB-MAS m/z 260 (M + +1)

元素分析値 (C₁₉H₂₅NO₄ · 1/4H₂Oとして)

計算値 (%) C:59.18 H:9.74 N:5.31

実測値 (%) C:58.89 H:9.77 N:5.29

実施例21 N-(3'-メチルチオプロピル)-1-デオキシ
ガラクトスタチンの合成

ホルマリンの代わりにメチルチオプロピオンアルデヒドを用いて実施例1と同様にして合成した。融点 121~124℃。

$[\alpha]_D$ -16.00 (20℃、C=0.550, H₂O)

FAB-MAS m/z 252 (M + +1)

元素分析値 (C₁₀H₂₁NO₄S · 1/2H₂Oとして)

計算値 (%) C:46.13 H:8.51 N:5.38

実測値 (%) C:46.11 H:8.21 N:5.30

実施例22 N-(3'-メチル-4'-メトキシベンジル)-1-
デオキシガラクトスタチンの合成

ホルマリンの代わりに3-メチル-4-メトキシベンズアルデヒドを用いて実施例1と同様にして合成した。融点 185~187℃。

FAB-MAS m/z 298 (M + +1)

元素分析値 (C₁₅H₂₃NO₅として)

計算値 (%) C:60.59 H:7.80 N:4.71

実測値 (%) C:60.48 H:7.82 N:4.66

実施例23 N-n-ブチル-1-デオキシガラクトスタチンの
合成

アリルブロマイドの代わりにn-ブチルブロマイドを用いて実施例9と同様にして合成した。融点 121~123℃。

$[\alpha]_D$ -23.90 (20℃、C=0.502, H₂O)

FAB-MAS m/z 220 (M + +1)

元素分析値 ($C_{10}H_{21}NO_4$ として)

計算値 (%) C:54.77 H:9.65 N:6.39

実測値 (%) C:54.33 H:9.56 N:6.36

実施例24 N-(3-カルボキシプロピル)-1-デオキシ
ガラクトスタチンの合成

アリルブロマイドの代わりに3-ブロモプロピオン酸エチルを用いて実施例9と同様にして合成した。融点 105~107℃。

$[\alpha]_D$ 8.10 (20℃、C=0.148, H_2O)

PAB-MAS m/z 250 ($M + +1$)

元素分析値 ($C_{10}H_{19}NO_6 \cdot H_2O$ として)

計算値 (%) C:44.94 H:7.92 N:5.24

実測値 (%) C:44.58 H:7.97 N:5.24

試験例

β -ガラクトシダーゼ阻害活性

0-ニトロフェニル- β -D-ガラクトピラノースを基質として β -ガラクトシダーゼを作用せしめ、加水分解されて遊離する0-ニトロフェノールを比色法で定量することにより測定した。即ち、100mM酢酸緩衝液 0.9ml (pH 5.0)、検体を含む溶液 (100mM酢酸緩衝液 pH 5.0に溶解) 0.1ml及び20mM基質溶液 0.5mlの混液を37℃で5分間予備加温した後、10mM酢酸緩衝液 pH 5.0に溶かした。次いで、 β -ガラクトシダーゼ溶液 0.5mlを加え、37℃で15分間反応した。そして、420nmにおける吸光度 (A) を測定し、同時に検体を含まない反応液の吸光度 (B) を測定し、阻害率を $(B - A) / B \times$

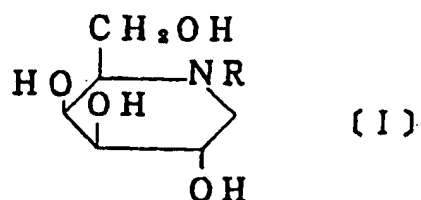
100により算出し、 β -ガラクトシダーゼ活性を50%阻害する濃度 (IC_{50}) を求めた。2回行った試験 (試験例1と試験例2) の結果を表1に示す。なお、 β -ガラクトシダーゼはアスペルギルス属 (*Aspergillus* sp.) 由来のものを用いた。本発明化合物が、強い β -ガラクトシダーゼ阻害活性を有していることが明らかである。

表1 β -ガラクトシダーゼ阻害活性

試験例 1		試験例 2	
実施例 番号	IC_{50} (ng/ ml)	実施例 番号	IC_{50} (ng/ ml)
1	15	11	59
2	82	12	166
3	44	13	113
4	40	14	391
5	32	15	393
6	23	16	21
7	88	17	49
8	180	18	340
9	187	20	37
10	175	21	49
対照 (1-デオキシ ガラクトスタチン 塩酸塩)	451	22	359
		23	154
		24	351
		対照 (1-デオキシ ガラクトスタチン 塩酸塩)	440

請求の範囲

1. 次の一般式〔I〕



(式中 R は、直鎖状、分枝状若しくは環状の置換基を有していてもよい炭素数 1～18 の飽和炭化水素又は不飽和炭化水素を示す。) で表される 3, 4, 5-トリヒドロキシピペリジン誘導体又はその薬理学的に許容される塩。

INTERNATIONAL SEARCH REPORT

International Application No. PCT/JP91/00866

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ¹ According to International Patent Classification (IPC) or to both National Classification and IPC <div style="text-align: center; padding: 10px;">Int. Cl⁵ C07D211/46, A61K31/445</div>																	
II. FIELDS SEARCHED <div style="text-align: center; padding: 5px;">Minimum Documentation Searched ²</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 30%; padding: 5px;">Classification System</th> <th style="padding: 5px;">Classification Symbols</th> </tr> <tr> <td style="text-align: center; padding: 10px;">IPC</td> <td style="padding: 10px;">C07D211/46, A61K31/445</td> </tr> </table> <div style="text-align: center; padding: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ³</div>			Classification System	Classification Symbols	IPC	C07D211/46, A61K31/445											
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IPC	C07D211/46, A61K31/445																
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁴ <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; padding: 5px;">Category ⁵</th> <th style="width: 60%; padding: 5px;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 30%; padding: 5px;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; padding: 10px;">A</td> <td style="padding: 10px;">JP, A, 62-242663 (Bayer AG), October 23, 1987 (23. 10. 87) & EP, A2, 240,868</td> <td style="text-align: center; padding: 10px;">1</td> </tr> <tr> <td style="text-align: center; padding: 10px;">A</td> <td style="padding: 10px;">JP, A, 61-200967 (Bayer AG), September 5, 1986 (05. 09. 86) & EP, A2, 193,770</td> <td style="text-align: center; padding: 10px;">1</td> </tr> <tr> <td style="text-align: center; padding: 10px;">A</td> <td style="padding: 10px;">JP, A, 57-134465 (Bayer AG), August 19, 1982 (19. 08. 82) & EP, A1, 55431</td> <td style="text-align: center; padding: 10px;">1</td> </tr> <tr> <td style="text-align: center; padding: 10px;">A</td> <td style="padding: 10px;">JP, A, 64-31764 (Bayer AG), February 2, 1989 (02. 02. 89) & EP, A2, 298,350</td> <td style="text-align: center; padding: 10px;">1</td> </tr> </table>			Category ⁵	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	A	JP, A, 62-242663 (Bayer AG), October 23, 1987 (23. 10. 87) & EP, A2, 240,868	1	A	JP, A, 61-200967 (Bayer AG), September 5, 1986 (05. 09. 86) & EP, A2, 193,770	1	A	JP, A, 57-134465 (Bayer AG), August 19, 1982 (19. 08. 82) & EP, A1, 55431	1	A	JP, A, 64-31764 (Bayer AG), February 2, 1989 (02. 02. 89) & EP, A2, 298,350	1
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>																	
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search <div style="text-align: center; padding: 10px;">August 30, 1991 (30. 08. 91)</div> </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report <div style="text-align: center; padding: 10px;">September 17, 1991 (17. 09. 91)</div> </td> </tr> <tr> <td style="padding: 5px;"> International Searching Authority <div style="text-align: center; padding: 10px;">Japanese Patent Office</div> </td> <td style="padding: 5px;"> Signature of Authorized Officer </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; padding: 10px;">August 30, 1991 (30. 08. 91)</div>	Date of Mailing of this International Search Report <div style="text-align: center; padding: 10px;">September 17, 1991 (17. 09. 91)</div>	International Searching Authority <div style="text-align: center; padding: 10px;">Japanese Patent Office</div>	Signature of Authorized Officer											
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国 際 調 査 報 告

国際出願番号PCT/JP 91/ 00866

I. 発明の属する分野の分類		
国際特許分類 (IPC)		
Int. Cl. C07D211/46, A61K81/445		
II. 国際調査を行った分野		
調 査 を 行 っ た 最 小 限 資 料		
分類体系	分類記号	
IPC	C07D211/46, A61K81/445	
最小限資料以外の資料で調査を行ったもの		
III. 関連する技術に関する文献		
引用文献の カテゴリー	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	請求の範囲の番号
A	JP, A, 62-242668 (パイエル・アクチエンゲゼル シャフト), 28. 10月. 1987 (28. 10. 87) &EP, A2, 240868	1
A	JP, A, 61-200967 (パイエル・アクチエンゲゼル シャフト), 5. 9月. 1986 (05. 09. 86) &EP, A2, 193770	1
A	JP, A, 57-184465 (パイエル・アクチエンゲゼル シャフト), 19. 8月. 1982 (19. 08. 82) &EP, A1, 55481	1
A	JP, A, 64-81764 (パイエル・アクチエンゲゼル	1
<p>※引用文献のカテゴリー</p> <p>「A」特に関連のある文献ではなく、一般的技術水準を示すもの</p> <p>「E」先行文献ではあるが、国際出願日以後に公表されたもの</p> <p>「L」優先権主張に疑義を提起する文献又は他の文献の発行日 若しくは他の特別な理由を確立するために引用する文献 (理由を付す)</p> <p>「O」口頭による開示、使用、展示等に言及する文献</p> <p>「P」国際出願日前で、かつ優先権の主張の基礎となる出願の 日の後に公表された文献</p> <p>「T」国際出願日又は優先日の後に公表された文献であって出 願と矛盾するものではなく、発明の原理又は理論の理解 のために引用するもの</p> <p>「X」特に関連のある文献であって、当該文献のみで発明の新 規性又は進歩性がないと考えられるもの</p> <p>「Y」特に関連のある文献であって、当該文献と他の1以上の 文献との、当業者にとって自明である組合せによって進 歩性がないと考えられるもの</p> <p>「&」同一パテントファミリーの文献</p>		
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80. 08. 91	17.09.91	
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第2ページから続く情報	
<p>(頁欄の続き)</p> <p>シャフト), 2. 2月. 1989(02. 02. 89) &EP, A2, 398,850</p>	
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(51) International Patent Classification ⁶ : A61K 31/445, C07D 211/46	A1	(11) International Publication Number: WO 98/02161 (43) International Publication Date: 22 January 1998 (22.01.98)
(21) International Application Number: PCT/NL97/00411 (22) International Filing Date: 14 July 1997 (14.07.97) (30) Priority Data: 96202010.3 15 July 1996 (15.07.96) EP (34) Countries for which the regional or international application was filed: NL et al. (71) Applicant (for all designated States except US): UNIVERSITEIT VAN AMSTERDAM [NL/NL]; Meibergdreef 15, NL-1105 AZ Amsterdam (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): AERTS, Johannes, Maria, Franciscus, Gerardus [NL/NL]; Universiteit van Amsterdam, Meibergdreef 15, NL-1105 AZ Amsterdam (NL). PANDIT, Upendra, Kumar [NL/NL]; Universiteit van Amsterdam, Meibergdreef 15, NL-1105 AZ Amsterdam (NL). KOOMEN, Gerrit, Jan [NL/NL]; Universiteit van Amsterdam, Meibergdreef 15, NL-1105 AZ Amsterdam (NL). OVERKLEEF, Herman, Steven [NL/NL]; Universiteit van Amsterdam, Meibergdreef 15, NL-1105 AZ Amsterdam (NL). VIANELLO, Paola [IT/IT]; Universiteit van Amsterdam, Meibergdreef 15, NL-1105 AZ Amsterdam (NL).	(74) Agent: SMULDERS, Th., A., H., J.; Verenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: DEOXYNOJIRIMYCIN DERIVATIVES AND THEIR USES AS GLUCOSYLCERAMIDASE INHIBITORS (57) Abstract Deoxynojirimycin derivatives containing a large hydrophobic moiety, such as cholesterol or adamantane-methanol, linked through a spacer, such as pentamethylene, to the nitrogen atom of deoxynojirimycin, and salts thereof, inhibit glucosylceramidase and may be useful in the treatment of diseases involving a ceramide-mediated signalling process, such as Gaucher disease.		

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DEOXYNOJIRIMYCIN DERIVATIVES AND THEIR USES AS GLUCOSYLCERAMIDASE INHIBITORS**FIELD OF THE INVENTION**

This invention is in the fields of therapy and pharmaceutical compositions for the treatment of various diseases, in particular diseases characterized by elevated plasma chitotriosidase levels, such as Gaucher disease.

BACKGROUND OF THE INVENTION**CERAMIDE, A SECOND MESSENGER**

In recent years the importance of ceramide as second messenger in signal transduction has been recognized. It has become clear that the signalling induced by a number of cytokines is mediated by changes in the intracellular concentration of this lipid [1,2]. For example, crucial for the transduction of the signal exerted by TNF- α (tumor necrosis factor alpha) upon binding to its receptor are local changes in ceramide concentration in specific regions, or invaginations, of the plasma membrane. Upon binding of the cytokine to its receptor, a sphingomyelinase catalyzes the conversion of sphingomyelin into phosphorylcholine and ceramide. The ceramide that is generated in this manner propagates the signal by activating specific protein kinases and phosphatases, resulting in a cellular response. Fig. 1 gives an overview of the signalling mechanism of TNF- α and other cytokines such as interferon gamma and interleukin 6.

There is now convincing experimental evidence for the role of ceramide in signalling. It has been shown that the effects of TNF- α can be experimentally mimicked by administration of a permeable ceramide with truncated fatty acyl moiety or, alternatively, by the generation of ceramide at the cell surface by the treatment of cells with a bacterial sphingomyelinase (see e.g. ref. 2).

The above described signal transduction process is most likely a highly local event, occurring near the cytokine

receptor. The concentration of ceramide in the plasma membrane is believed to be very low under normal conditions. However, considerable amounts of ceramide are present in the plasma membrane as a building block in sphingomyelin. The hydrolysis of sphingomyelin would allow a considerable local change in ceramide concentration and subsequent signal propagation.

Via action of a specific transferase, ceramide can be reconverted to sphingomyelin by transfer of the phosphorylcholine moiety from phosphatidylcholine (PC), resulting in the concomitant formation of diacylglycerol. The total pathway, resulting in the netto hydrolysis of phosphatidylcholine to phosphorylcholine and diacylglycerol, is named the sphingomyelin cycle [2].

15

CERAMIDE AND SPHINGOLIPID METABOLISM

Obviously, not all fluctuations in intracellular ceramide concentrations are affecting signal transduction. Ceramide is extensively metabolized in cells. The lipid is synthesized at the membrane of the endoplasmic reticulum from acylCoA and sphingosine. It may be converted at the level of the Golgi apparatus into sphingomyelin, glucosylceramide and related complex gangliosides, or galactosylceramide and related globosides and sulfatides. Sphingomyelin and glycosphingolipids are also catabolized into ceramide and other components in the lysosomal compartment of cells. The intralysosomally formed ceramide may be locally hydrolyzed into sphingosine and fatty acid by the action of the lysosomal ceramidase or it may be exported to the cytosol and re-used for synthesis of sphingolipids. A schematic overview of the ceramide metabolism is presented in Fig. 2.

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SPHINGOLIPIDOSES: GAUCHER DISEASE

In man a number of inherited disorders in lysosomal sphingolipid catabolism occur, the so called sphingolipidoses (see Table 1). For example, an inherited deficiency of the

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lysosomal sphingomyelinase underlies Niemann-Pick disease, and defective activity of the lysosomal ceramidase causes Farber disease. The most frequently encountered sphingolipidosis is Gaucher disease [3]. The metabolic basis of this disorder is a deficiency of the lysosomal beta-glucosidase, glucocerebrosidase (E.C.3.2.1.45). This enzyme catalyzes the hydrolysis of glucosylceramide (glucocerebroside) to glucose and ceramide. In patients with Gaucher disease glucosylceramide accumulates in tubular aggregates, in particular in lysosomes of macrophages. The lipid-laden macrophages have a typical morphology and are usually referred to as 'Gaucher cells'. In the course of clinical manifestation of Gaucher disease the abnormal macrophages may accumulate in large quantities in various body locations, such as the bone marrow compartment, spleen, liver, kidney, and lungs. The most pronounced clinical symptoms associated with Gaucher disease are progressive splenomegaly, hepatomegaly, and skeletal deterioration. Most Gaucher disease patients do not develop neurological complications. The common non-neuronopathic form of the disease is called Type 1 Gaucher disease. In very severe cases of Gaucher disease characteristic neurological abnormalities may also occur, resulting in lethal complications at infantile (Type 2) or juvenile (Type 3) age [3].

25 GAUCHER CELLS

The glucosylceramide-laden Gaucher cells are believed to play a crucial role in the pathophysiology. Their massive presence in various body locations is thought to lead to local pathology.

30 Gaucher cells should not be viewed as inert containers of glycosphingolipid. The storage cells are viable and actually, being activated macrophages, secrete large amounts of specific proteins such as hydrolases and cytokines. These factors in turn act as pathogenetic agents that cause local tissue damage and turnover. Moreover, Gaucher-cell derived factors such as cytokines promote the recruitment of

additional activated macrophages (see Fig. 3 for a schematic overview).

Recently a sensitive marker for Gaucher cells has been discovered by us [4]. Using the technique of in situ hybridization we observed that Gaucher cells synthesize large quantities of the secretory enzyme chitotriosidase, the human analogue of chitinases present in various species. This explains the dramatic elevation in plasma chitotriosidase levels in clinically affected Gaucher patients. On the average chitotriosidase levels are about 1000 fold higher in plasma of these patients as compared to corresponding normal subjects. In presymptomatic or asymptomatic individuals with an inherited glucocerebrosidase deficiency plasma chitotriosidase levels are (almost) within the normal range (see Table 2). Interestingly, elevated levels of plasma chitotriosidase have also been noted for patients with other sphingolipidoses, in particular Niemann-Pick disease [5].

It has been observed that in cultured macrophages, derived from peripheral blood monocytes, the concentration of glucosylceramide gradually increases. The increase in glycolipid is more pronounced when cells are grown in the presence of conduritol B-epoxide, a potent irreversible inhibitor of glucocerebrosidase. After approximately 7 days of culture the macrophages start to produce chitotriosidase mRNA and secrete the enzyme [4,6]. The expression of the chitotriosidase gene subsequently dramatically increases: after about three weeks chitotriosidase constitutes almost 1% of the total synthesized protein, as revealed by the incorporation of radioactively labeled methionine [7]. The continuous presence in the culture medium of glucosylceramide, or of conduritol B-epoxide (an irreversible inhibitor of lysosomal glucocerebrosidase), promotes chitotriosidase expression.

THERAPEUTIC INTERVENTION FOR GAUCHER DISEASE

The sparse and anecdotal information on the natural history of Gaucher disease indicates that although clinical symptoms develop progressively, the disease manifestation is usually not a gradual process. In the case of most patients abnormalities develop rapidly at a particular age in a specific tissue, may subsequently stabilize for considerable time, to become next rapidly progressive again. In other words, disease progression has a local and chaotic feature. Most likely, Gaucher cells play a critical role in these local pathogenetic processes. The presence of the activated storage cells will locally induce tissue damage and turnover, and promote recruitment of activated macrophages at these sites, initiating a chaotic cascade of pathological events (see Fig. 3). According to this concept, a major beneficial effect should be exerted by a disruption or prevention of the pathological cascade. The various therapeutic approaches for Gaucher disease that have been considered are discussed here below.

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ENZYME SUPPLEMENTATION THERAPY

For more than thirty years supplementation of macrophages of Gaucher patients with human glucocerebrosidase has been seriously considered as a therapeutic option. Efforts to develop a therapy for Gaucher disease have been largely unsuccessful for many years due to the unavailability of sufficient amounts of pure human glucocerebrosidase and the poor targeting of intravenously administered enzyme to lysosomes of tissue macrophages. Only since 1990 an effective therapeutic intervention for Gaucher disease is available that is based on the chronic supplementation of patients with human glucocerebrosidase [8]. Administered by intravenous infusion is a human glucocerebrosidase that is modified in its N-linked glycans such that mannose-residues are terminally exposed. The modification favours uptake via mannose receptors. Improved targeting of the modified

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('mannose-terminated') enzyme to lysosomes of tissue macrophages occurs via mannose-receptor mediated endocytosis. Different dosing regimens that vary with respect to total dose (15-240 U/kg body weight.month) and frequency of administration (three times weekly to biweekly) are presently used (see e.g. ref. 9). Glucocerebrosidase isolated from human placenta (Ceredase; alglucerase) and enzyme recombinantly produced in CHO-cells (Cerezyme; imiglucerase) have been found to be equally potent in reversing some of the clinical signs associated with the disease [10].

The most pronounced beneficial effects of enzyme replacement therapy are the reductions in liver and spleen volumes, and the improvements in hematological parameters such as hemoglobin concentration and thrombocyte and leukocyte counts. Marked interindividual differences exist in the rate and extent of clinical response, even among related patients that are treated with the same dosing regimen [9]. In general, the most marked clinical improvements occur within the first year of treatment, accompanied by a pronounced correction of biochemical serum abnormalities. A complete reversal of clinical signs and complete normalization of serum abnormalities, such as elevated levels of angiotensin converting enzyme, tartrate-resistant acid phosphatase and chitotriosidase, is not accomplished by enzyme therapy, not even in the case of patients that receive a high dose of glucocerebrosidase for a number of years [11]. The partial correction following enzyme therapy is in contrast to the complete correction that is noted for patients that underwent a successful bone marrow transplantation.

Conflicting views still exist with respect to the optimal dosing regimen for enzyme therapy. Whereas low dosing regimens may be (almost) equally successful to high dosing regimens in generating hematological improvements, this is still questionable with respect to intervention of the bone disease.

Presently more than 1500 patients are receiving enzyme therapy. This recent development has attracted considerable scientific and public attention, also due to the high costs and potential risks that are involved. The costs associated with successful therapy have hitherto been exceptionally high (\$100,000 to \$400,000 annually per patient); leading to the belief that the enzyme therapy of Gaucher disease is the most expensive drug treatment for any disease. Although the alglucerase preparation is known to contain minor amounts of HCG and other impurities, the experience so far indicates that enzyme therapy is safe.

The enzyme therapy for Gaucher disease is considered to be a model case for the future development of treatments for other rare genetic disorders - a point perhaps best illustrated by the organisation in February/March 1995 of a Technology Assessment Conference at the National Institutes of Health, Bethesda, USA, that was specifically devoted to Gaucher disease. This type of conference is only organised when there is an exceptionally pressing health care need. During the conduct of the conference, a panel of 12 independent experts took evidence from leading scientists and clinicians in the field of Gaucher disease; the panel concluded that enzyme therapy is effective in reversing a number of clinical signs associated with Gaucher disease. Furthermore, it was stressed that reduction of the costs and the associated potential risks of human protein replacement therapy are critical issues both from the point of view of patient care and health care economics [12].

OTHER THERAPEUTIC APPROACHES

A successful treatment of Gaucher disease by bone marrow transplantation has been accomplished for a limited number of juvenile Gaucher patients. The introduction of the normal genetic information for glucocerebrosidase in hematopoietic stem cells results in the formation of blood cells that are able to hydrolyze glucosylceramide at normal rates. The fact

that clinical abnormalities disappear in Gaucher patients following a successful bone marrow transplantation indicates that the presence of blood cells with normal glucocerebrosidase activity is sufficient for prevention of disease symptoms. Unfortunately, the applicability of bone marrow transplantation as treatment for Gaucher disease is quite restricted due to the limited availability of bone marrow from matched donors and the considerable morbidity associated with this intervention, particularly in the case of adults.

10 In recent years the option of gene therapy of Gaucher disease is intensively studied. In general, the following approach is envisioned. Pluripotent hematopoietic stem cells are isolated and transduced with a vector containing human glucocerebrosidase cDNA. After successful transduction the stem cells are re-introduced in the patient. Although data obtained with animal studies suggest that Gaucher disease is an attractive candidate for gene therapy, a number of serious problems still have to be solved before efficient intervention in this manner can be expected. A major disadvantage is that the 'genetically corrected' stem cells and their progeny most likely have no selective advantage. It is therefore assumed that in order to be effective gene therapy has to result in a stable correction of a major proportion of the pluripotent stem cells. For a critical evaluation of the state of the art concerning gene therapy see ref. 13.

25 A distinct therapeutic approach that has been proposed for Gaucher disease is the so called 'substrate deprivation therapy' [14-16]. It is argued that a marked reduction of the synthesis of glucosylceramide may have a beneficial effect because the amount of glucosylceramide that has to be degraded by macrophages would be lower. Several inhibitors of glucosylceramide synthase have been developed, e.g. 1-phenyl-decanoylamino-3-morpholino-1-propanol (PDMP) and its analogue 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP) [14], butyl-deoxynojirimycin [15] and butyl-deoxygalactonojirimycin [16].

A disadvantage of the 'substrate deprivation' approach is that a priori not only the synthesis of glucosylceramide but also that of more complex glycosphingolipids is inhibited. Moreover, the presently available inhibitors of glucosylceramide synthase are known to exert a number of important biological effects that may limit their applicability as therapeutic agent. For example, PDMP is known to induce apoptosis in some cell types. Butyl-deoxynojirimycin is known to inhibit also the lysosomal glucocerebrosidase and the α -glucosidase I, an ER enzyme that plays a critical role in trimming of N-linked glycans in newly formed glycoproteins and as such in quality control of protein folding. The antiviral action of butyl-deoxynojirimycin is thought to be caused by its inhibitory effect on glycoprotein modification. Moreover, it was recently reported that glucosylceramide synthase inhibitors induce the synthesis of the enzyme. Consequently, these inhibitors would need to be chronically administered to Gaucher patients since their withdrawal would be followed by an abnormally high level of glucosylceramide synthase activity and increased load on glucosylceramide [14].

SUMMARY OF THE INVENTION

This invention provides a deoxynojirimycin derivative containing a large hydrophobic moiety linked through a spacer to the nitrogen atom of deoxynojirimycin, and salts thereof.

The word 'spacer' refers to any bivalent moiety or group capable of linking a hydrophobic group to the N atom of deoxynojirimycin. Said spacer preferably comprises a polyalkylene chain of from 3 to 8 carbon atoms, more preferably 3 to 6 carbon atoms, most preferably 5 carbon atoms. In a particularly preferred embodiment of the invention, the spacer consists of a group having the structure $-(CH_2)_n-$ wherein n is an integer from 3 to 8, preferably 3 to 6, most preferably 5.

The phrase 'large hydrophobic moiety' refers to any hydrophobic group or moiety that has a lipophilic nature and tends to stably insert in biological lipid-bilayer membranes. Normally it comprises at least one saturated, unsaturated or partially unsaturated cyclic structure, in particular a condensed ring structure comprising two or more condensed rings. More preferably, the large hydrophobic moiety is derived from a polycyclic alcohol containing three or more rings each sharing two or more carbon atoms with another ring. The large hydrophobic moiety has the ability to insert in lipid bilayers.

Preferably said large hydrophobic moiety is derived from a compound selected from the group consisting of adamantane-methanol, cholesterol, β -cholestanol, adamantanol and 9-hydroxyphenanthrene.

This invention furthermore provides a pharmaceutical composition containing such deoxynojirimycin derivative, and a variety of applications of said deoxynojirimycin derivative, including several therapeutical uses.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 presents a schematic overview of signalling through ceramide. TNF- α (tumor necrosis factor alfa), IL-1 (interleukin 1), NGF (nerve growth factor), IFN (interferon gamma) bind to their receptors, whereupon a neutral sphingomyelinase generates ceramide from sphingomyelin. Ceramide activates protein kinases and phosphatase which results in a cellular response.

Figure 2 presents a schematic overview of metabolism of ceramide. Abbreviations used therein: Chol = choline; Glc = glucose; GlcCer = glucosylceramide; GSL = complex glycosphingolipid; LacCer = lactosylceramide; SM = sphingomyelin.

Figure 3 depicts the pathophysiology of Gaucher disease. Lipid-laden macrophages ('Gaucher cells') secrete hydrolases and cytokines that cause tissue damage and turnover and that

promote recruitment of macrophages, thus causing a pathological cascade.

Figure 4 presents a hypothetical model for the pathogenesis of Gaucher disease and a target for intervention. Due to lysosomal impairment of GC (glucosylceramide) catabolism in the lysosomes the activity of the non-lysosomal glucosylceramidase is increased. This results in increased ceramide (C) production and associated therewith signalling to the nucleus. This leads to production and secretion of specific factors that propagate the pathological cascade. Intervention in the pathogenetic mechanism should be feasible by specifically inhibiting the activity of glucosylceramidase.

DETAILED DESCRIPTION OF THE INVENTION

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A NOVEL THERAPEUTIC APPROACH: INHIBITION OF MACROPHAGE ACTIVATION

Currently enormous costs are associated with enzyme therapy and the efficacy of this approach proves to be individually highly variable. The present alternatives for therapeutic intervention either can be applied only for a limited number of cases (bone marrow transplantation), or have in fact not yet been shown to be effective and safe (gene therapy and substrate deprivation). This has prompted us to search for a novel option for therapeutic intervention that may be used in addition to enzyme therapy.

According to our view on the pathogenesis of Gaucher disease (see Fig. 3), the activation of macrophages that leads to the release of hydrolases and cytokines forms an ideal target for intervention.

An agent that is capable of preventing the activation of Gaucher cells should have a therapeutic value, and moreover should be able to augment the efficacy of enzyme therapy.

THE DEVELOPMENT OF AN INTERVENTION BASED ON INHIBITION OF MACROPHAGE ACTIVATION

IDENTIFICATION OF THE TARGET FOR INTERVENTION

5 In order to be able to develop the desired therapeutic agent, first the mechanism by which Gaucher cells are driven in their characteristic activated state had to be elucidated.

 Two crucial findings were made by us in the course of our investigations that allowed us to develop the envisioned
10 agent.

 In the first place, we discovered a sensitive marker for the characteristic activated state of Gaucher cells, i.e. the massive synthesis and secretion of chitotriosidase by these cells (see above). Importantly, the potential of agents to
15 prevent the relevant activation of macrophages can be sensitively tested by the analysis of their capacity to inhibit the production and secretion of chitotriosidase by macrophages in cell culture.

 In the second place, we discovered that human cells
20 contain, besides the lysosomal glucocerebrosidase, a distinct enzyme that is capable of hydrolyzing glucosylceramide into glucose and ceramide [17].

NON-LYSOSOMAL GLUCOSYLCERAMIDASE

25 The glucosylceramidase differs in many respects from the lysosomal glucocerebrosidase. The enzyme is not located in lysosomes in contrast to glucocerebrosidase; it is not deficient in Gaucher disease in contrast to glucocerebrosidase; it behaves as an integral membrane protein whilst glucocerebrosidase shows the features of a membrane-associated
30 protein; and finally, it differs from glucocerebrosidase in specificity towards artificial substrates, inhibitors and activators. For example, the glucosylceramidase is not able to hydrolyze artificial b-xylosidic substrates contrary to
35 glucocerebrosidase. Glucocerebrosidase is irreversibly inhibitable by conduritol B-epoxide in contrast to the

glucosylceramidase that is insensitive for this compound. The lysosomal activator protein saposin C potently stimulates glucocerebrosidase in its enzymatic activity but is without effect on the glucosylceramidase.

5 Concerning the function of glucosylceramidase, an important observation was made. Using a relatively novel technique for subcellular fractionation it was found that the glucosylceramidase is present at the plasma membrane or in early endosomal structures. In other words, the enzymatic
10 activity of the glucosylceramidase results in the generation of ceramide in the plasma membrane or specific invaginations of this membrane. It is known that significant amounts of glucosylceramide are indeed present in the plasma membrane. Consequently the activity of glucosylceramidase might result
15 in relevant changes in ceramide concentration in those cellular membranes that are involved in ceramide-mediated signalling.

It was furthermore observed using membrane suspensions prepared from cells and tissues that the ceramide formed from
20 glucosylceramide by the activity of the lysosomal glucocerebrosidase is hardly converted into sphingomyelin; in sharp contrast to this is the efficient conversion of the ceramide formed by the action of glucosylceramidase into sphingomyelin. Apparently, the ceramide generated by the
25 glucosylceramidase activity is rapidly further metabolized within the same membranes, as can be expected for a lipid that acts as transient second messenger.

On the basis of these findings we postulate a new mechanism for the pathological activation of macrophages in
30 Gaucher disease. In this model, as depicted in Fig. 4, it is proposed that the lysosomal impairment to degrade glucosylceramide in glucocerebrosidase-deficient individuals also leads to an increased concentration of this glycolipid in membranes containing the glucosylceramidase. Consequently,
35 the glycolipid is there at an abnormal high rate hydrolysed to ceramide. This ceramide activates protein kinases and

phosphatases, resulting in the characteristic activation of the macrophage and the corresponding production and release of pathogenetic factors. Experimental proof for this model in which the constitutively stimulated glucosylceramidase activity promotes macrophage activation is described below.

GLUCOSYLCERAMIDASE ACTIVITY AS TARGET FOR THERAPEUTIC INTERVENTION

The glucosylceramidase is an ideal and novel target for prevention of the activation of macrophages in Gaucher disease. Specific inhibition of the enzyme activity would prevent further release of pathogenetic factors and disrupt the pathological cascade, resulting in a therapeutic effect. It may be envisioned that the combination of this approach with that of enzyme supplementation can improve markedly the efficacy of therapeutic intervention and meanwhile will result in a significant reduction of associated costs.

DESIGN OF A SPECIFIC INHIBITOR FOR GLUCOSYLCERAMIDASE ACTIVITY

The properties of the glucosylceramidase present in membrane suspensions and intact cells were carefully analysed in order to identify a suitable inhibitor for the enzyme. A number of important findings were made in this connection.

It was observed that the enzyme is tightly integrated in the membrane and most likely hydrolyzes its substrate glucosylceramide while this is also inserted in the membrane. The identification of the location of the glucosylceramidase in (invaginations of) the plasma membrane is also of importance.

Furthermore, a number of known glucosidase inhibitors (D-gluconolacton, castanospermine, deoxynojirimycin and butyl-deoxynojirimycin) were tested on their capacity to inhibit the glucosylceramidase activity. The most promising inhibitors were deoxynojirimycin and in particular butyl-deoxynojirimycin. However their specificity as well as that

of the other inhibitors tested was poor. For example, the lysosomal glucocerebrosidase is also quite sensitive to the inhibitors, rendering them unattractive for administration to the already glucocerebrosidase-deficient Gaucher patients.

5 The inhibitors would moreover seriously interfere with enzyme therapy of patients due to their inhibitory effect on the administered alglucerase or imiglucerase.

It was noted that incubation of intact cells with deoxynojirimycin or butyl-deoxynojirimycin at their IC₅₀ concentration for glucosylceramidase (20 and 0.3 μ M, respectively), resulted also in a significant inhibition of glucocerebrosidase activity (about 20 and 10%, resp.) and in an inhibition of glucosylceramide synthase activity (about 30 and 20%, respectively). In the same concentration range a marked inhibition of ER α -glucosidase I activity (N-linked glycan trimming) has also been reported for several cell types [15].

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The negative results with known glucosidase inhibitors prompted us to design a novel, more specific inhibitor for glucosylceramidase, exploiting the generated information on the features of the enzyme.

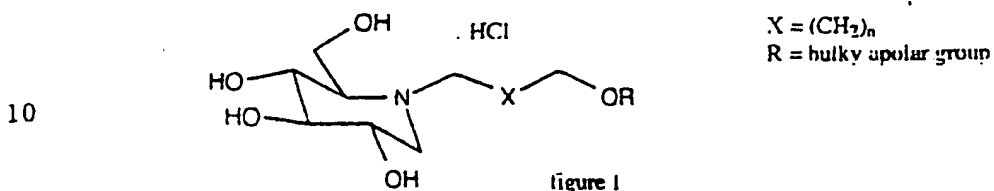
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It was conceived that the desired potent and specific inhibitor for the glucosylceramidase should have the following features:

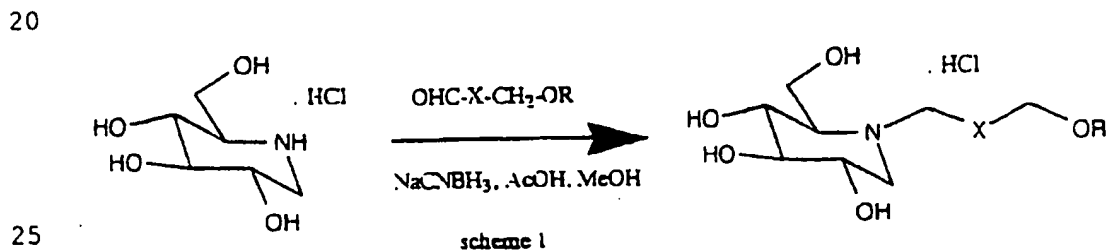
- 25 1- a deoxynojirimycin-moiety;
a proven, relatively potent inhibitor of the enzymatic activity of glucosylceramidase.
- 2- a N-alkyl spacer;
N-alkylation of deoxynojirimycin was found to increase its capacity to inhibit glucosylceramidase.
- 30 3- coupled to the spacer a large hydrophobic group that tends to insert in a lipid bilayer, preferably (invaginations of) the plasma membrane;
preferential insertion of the inhibitor in glucosylceramidase-containing membranes should increase the in vivo capacity and specificity of the inhibitor.
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SYNTHESIS OF DEOXYNOJIRIMYCIN-ANALOGUES

A series of deoxynojirimycin-derivatives was made by chemical synthesis in order to test the concept and develop the ideal inhibitor for the glucosylceramidase. Based on the abovementioned features, a series of deoxynojirimycin (DNM) derivatives of the following type were synthesized (figure 1):

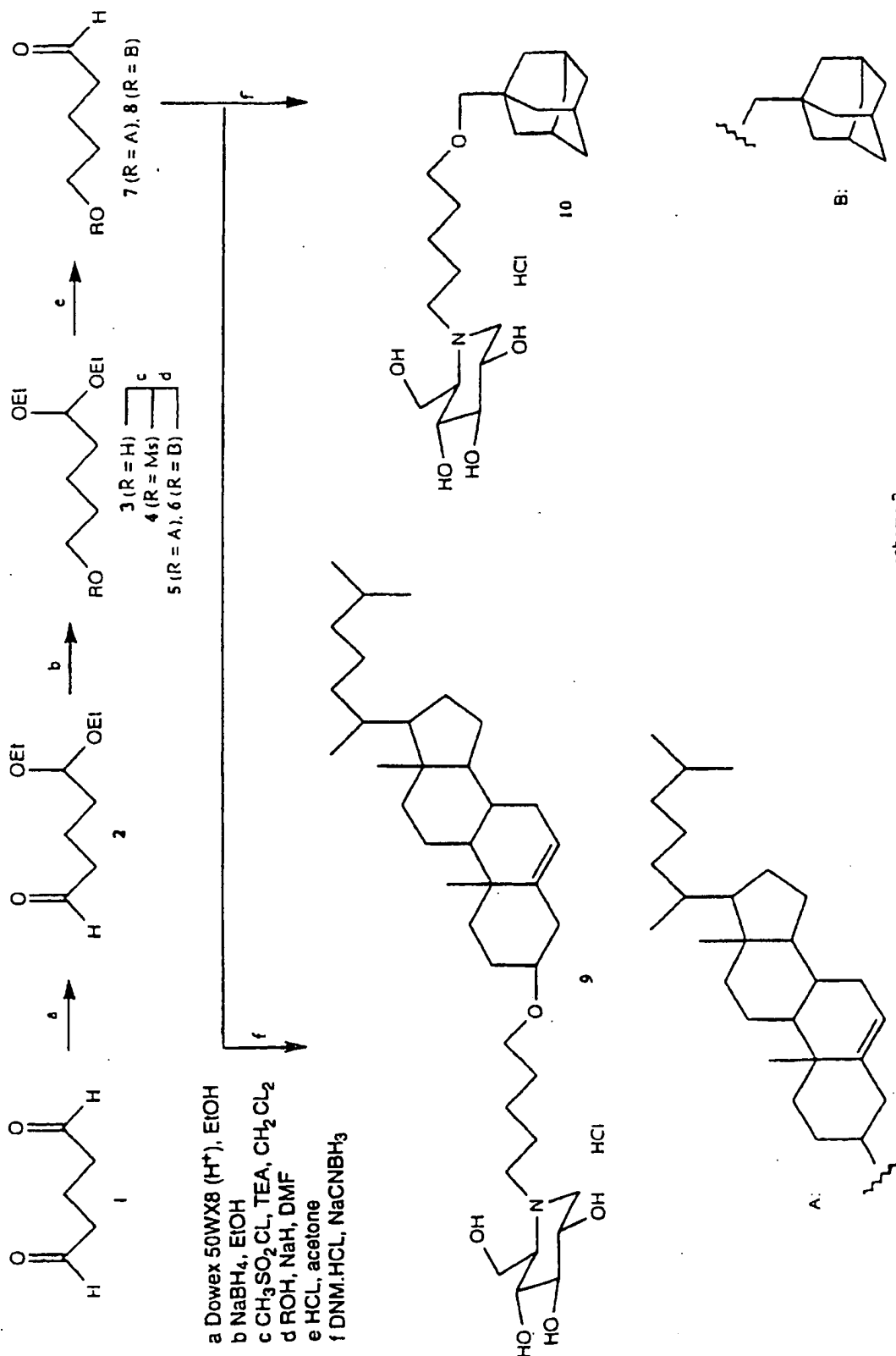


In this structure, X is a saturated alkane chain and R is a large apolar group. Compounds of this type can be synthesized by reacting DNM.HCl, which is readily available in seven steps from the commercially available tertabenzyl-glucopyranose [18], with the appropriate aldehyde, in a reductive amination [19] reaction (scheme 1).



The strategy is exemplified by the synthesis of the following two compounds: N-(5-cholesteroloxy-pentyl)-deoxynojirimycin 9 and N-(5-adamantane-1-yl-methoxy-pentyl)-deoxynojirimycin 10 (scheme 2). Thus, glutaric aldehyde 1 was first converted into the monoacetal 2 [20] using an ion exchange catalyst. After reduction of the monoacetal to the corresponding alcohol 3 and transformation to the mesylate 4, reaction with the appropriate alcohol, in which ROH is cholesterol and adamantanemethanol respectively, under basic

conditions, afforded the acetals 5 and 6. After liberation of the second aldehyde function, that is formation of compounds 7 and 8, reductive amination with DNM.HCl afforded the target compounds 9 and 10.



scheme 2

QUANTIFICATION OF THE INHIBITORY EFFECTS OF DEOXYNOJIRIMYCIN-ANALOGUES

The inhibitory effects of the various deoxynojirimycin-analogues on relevant enzyme activities were analysed in vitro and in intact cells.

IN VITRO EXPERIMENTS

Firstly, in vitro experiments were performed with purified human enzymes and membrane suspensions of human tissue. Attention was focussed to the inhibition of lysosomal glucocerebrosidase and glucosylceramidase activities and that of the lysosomal α -glucosidase. As source for glucosylceramidase were used a membrane fraction from human spleen. The glucosylceramidase activity was measured as the hydrolytic activity towards 4MU-b-glucoside in the membrane suspension that was pre-treated with conduritol B-epoxide to abolish the activity of glucocerebrosidase. As source of glucocerebrosidase served human placental enzyme (Ceredase, Genzyme Corp. Boston, USA) that is used in enzyme therapy. Alternatively, glucocerebrosidase activity was determined in a membrane fraction from human spleen. Glucocerebrosidase activity was measured as the hydrolysis of 4MU-b-glucoside that is inhibitable by conduritol B-epoxide. Lysosomal α -glucosidase activity was measured as the hydrolytic activity towards 4MU-a-glucosidase shown by a purified α -glucosidase preparation.

Tables 6 and 7 show the structures of the tested compounds. Table 3 gives an overview of the apparent K_i values of the inhibitors.

It can be seen in Table 3 that glucosylceramidase is potently inhibited by N-alkyl derivatives of deoxynojirimycin. Optimal inhibition was noted for the N-pentyl derivative. N-hexyl-deoxynojirimycin was a less potent inhibitor (not shown in Table 3). The presence of a carbonyl moiety in the spacer negatively influences the inhibitory capacity.

Coupling of a large hydrophobic group such as adamantane (P21) or cholesterol (P24) to a N-pentyl spacer dramatically increases the capacity of the compound to inhibit the glucosylceramidase activity.

5 IC₅₀ values were also determined in the case of some inhibitors. The apparent IC₅₀ values for P21 and P24 are extremely low, 1 nM and 0.1 μ M, respectively. For a comparison, the IC₅₀ values for deoxynojirimycin and butyl-deoxynojirimycin are 20 μ M and 0.3 μ M, respectively.

10 Table 3 shows that glucocerebrosidase is in general less sensitive to deoxynojirimycin derivatives than glucosylceramidase.

Pure glucocerebrosidase in solution (Ceredase) and enzyme associated to membranes show a different sensitivity for the inhibitors. Apparently, the kinetic properties of the

15 enzyme in these two different states differ, as is also suggested by the difference in apparent K_m for 4MU-b-glucoside.

Both the soluble and membrane-associated glucocerebrosidase are most potently inhibited by deoxynojirimycin-analogues with a N-pentyl spacer and coupled to it a large hydrophobic group.

For soluble glucocerebrosidase (Ceredase) the apparent IC₅₀ value of P21 was 0.2 μ M, and that of P24 was 0.8 μ M; for

25 the membrane-associated glucocerebrosidase the apparent IC₅₀ values of P21 and P24 were 0.06 and 0.7 μ M, resp.

With respect to the lysosomal α -glucosidase it was found that substitutions in deoxynojirimycin generally exerted relatively little effect. However, the compounds P4, P11,

30 P16, P9 and P13 were very poor inhibitors.

IN VIVO EXPERIMENTS

Next, the capacity of the deoxynojirimycin-analogues to inhibit the glucosylceramidase and the glucocerebrosidase activities in intact cells was investigated. Enzyme

35 activities were measured as described in ref. 17. Briefly,

the hydrolysis of 4MU-b-glucoside by cultured melanoma cells that were pre-incubated with and without conduritol B-epoxide was determined. The conduritol B-epoxide sensitive activity can be ascribed to glucocerebrosidase and the insensitive activity to glucosylceramidase. The results of this study are shown in Table 4.

A comparison of Table 3 and Table 4 reveals that the inhibition by deoxynojirimycin analogues of the glucosylceramidase activity in intact melanoma cells is similar to that observed in in vitro experiments using splenic membrane preparations. The most potent inhibitors are P21 and P24 with IC50 values of about 0.3 nM and 50 nM. At these or ten-fold higher concentrations no significant inhibition of the glucocerebrosidase activity is detectable, see Table 4.

The inhibitory constants of deoxynojirimycin analogues were also determined by analysis of the metabolism of C6-NBD glucosylceramide in melanoma cells, employing again conduritol B-epoxide to discriminate between the activities of the insensitive glucosylceramidase and the sensitive glucocerebrosidase. The results obtained with C6-NBD glucosylceramide as substrate were almost identical to those obtained with the fluorogenic 4MU-b-glucoside substrate (not shown).

It was studied to which extent other reactions were inhibited by incubating cells with P21 or P24 at their IC50 concentration for the glucosylceramidase activity. Under these conditions no inhibition of glycogen synthase was noted in rat hepatocytes; no inhibition of glucosylceramide synthase activity or lysosomal α -glucosidase was noted in cultured melanoma cells.

Because of the extreme sensitivity of the glucosylceramidase for P21, it was examined whether the inhibition by this compound might be not reversible. To test this, melanoma cells were preincubated with or without P21, and subsequently washed extensively. Next, the glucocerebrosidase and glucosylceramidase activities were determined with 4MU-b-

glucoside as substrate. It was found that the pretreatment with inhibitor was without significant effect on the glucocerebrosidase activity, but led to an irreversible loss of the glucosylceramidase activity.

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PROOF OF CONCEPT: VALUE OF DEOXYNOJIRIMYCIN ANALOGUES FOR INTERVENTION IN MACROPHAGE ACTIVATION

The effect of P21 (N-(5-adamantane-1-yl-methoxy-pentyl) deoxynojirimycin) and butyldeoxynojirimycin on macrophages in culture was examined. The iminosugars, dissolved in DMSO at a concentration of 10 mM, were added to cultured macrophages at various concentrations by dilution in culture medium. It was checked that the minor amounts of DMSO introduced in this manner were without effect.

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Table 5 shows the inhibition by the deoxynojirimycin analogues of the glucocerebrosidase and glucosylceramidase activities in macrophages, as measured with C6-NBD glucosylceramide as substrate; the effects are quite comparable to those noted for the enzymes in melanoma cells. Table 5 shows furthermore the effect of the deoxynojirimycin analogues on the secretion of chitotriosidase by the cells. It can be seen that chitotriosidase secretion is reduced concomitantly with inhibition of the activity of glucosylceramidase, but not of that of the lysosomal glucocerebrosidase. Using C6-NBD ceramide as substrate, glucosylceramide synthase activity in cultured macrophages was also determined. It was noted that this enzyme activity is not significantly inhibited by the presence of 5 uM butyldeoxynojirimycin in the culture medium, a condition causing a reduced chitotriosidase secretion. Moreover it was found that inhibition of glucosylceramide synthase by the presence of PDMP or PPMP was without effect on chitotriosidase secretion.

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In conclusion, the experiments show that low concentrations of butyldeoxynojirimycin and particularly of P21 are able, by virtue of their specific inhibition of glucosylceramidase activity, to de-activate macrophages that

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massively secrete chitotriosidase (and concomitantly other hydrolases and cytokines). Thus, experimental proof of concept has been obtained.

5 APPLICATIONS OF THE INHIBITORS OF GLUCOSYLCERAMIDASE ACTIVITY

One application for the newly developed, highly specific inhibitors is to be found in therapeutic intervention of Gaucher disease. As discussed above, the effects of the inhibitors on macrophage activation may be expected to
10 favourably interfere with the pathogenesis of Gaucher disease. The administration of inhibitors may improve the efficacy of enzyme therapy, and consequently result in an improved clinical response and a marked reduction of associated costs.

15 A beneficial effect might also be exerted by inhibitors of glucosylceramidase activity in the case of other disease states that are characterized by elevated plasma chitotriosidase, such as the Niemann-Pick disease and sarcoidosis [4,5]. Furthermore it is known to us that foam cells in
20 atherosclerosis are over-producing chitotriosidase.

It is likely that ceramide-mediated signalling processes are directly or indirectly effected by inhibition of glucosylceramidase activity. The potential applications for the developed inhibitors can therefore be extremely diverse.
25 Of particular interest are inflammatory states that are provoked by TNF- α or other proinflammatory cytokines that signal through ceramide. Examples in this connection are septic shock, rheumatoid arthritis and Crohn's disease.

The selection of a suitable route of administration and
30 suitable formulations of pharmaceutical compositions is within the normal skills of the persons skilled in the art. Examples of suitable administration routes are parenteral (intravenous, subcutaneous, intramuscular) injections or infusions, oral ingestion, and topical application. In
35 particular attractive is the use of oily vehicles allowing slow and sustained release from the repository preparation.

The use of an oily vehicle will not be feasible in the case of oral ingestion or intravenous administration. In the case of oral ingestion, absorption of the lipophilic compound will occur spontaneously in the gastro-intestinal tract upon the solubilization of the compound in mixed micelles followed by passive diffusion across the enterocyte membrane. In the case of intravenous administration use can be made of liposomes in which the lipophilic compound has prior been incorporated.

10 EXPERIMENTAL

5,5-Diethoxy-pentan-1-ol 3

A mixture of 5,5-diethoxy-pentanal 1 (3g, 17 mmol) and NaBH₄ (0.65 g, 17 mmol) in 30 ml EtOH was stirred at room temperature for 3 h. The solvent was evaporated and the residue was triturated with 10% NaOH and extracted with CH₂Cl₂. The organic layers were collected, dried (Na₂SO₄) and the solvent evaporated to give 2 purified by silica gel flash chromatography eluting with petroleum ether 60-80/ ethyl acetate 1:1 (yield 59%).

¹H NMR (CDCl₃): δ 4.47 (t, 1H, J= 5.7 Hz, C-1), 3.70-3.58 (m, 4H, C-5, CH₂ acetal), 3.46 (dq, 2H, J= 7.1 Hz, 2.3 Hz, CH₂ acetal), 1.65-1.50 (m, 4H, C-2, C-4), 1.41 (m, 2H, C-3), 1.18 (t, 6H, J=7.1 Hz, CH₃ acetal).

Methanesulfonic acid 5,5-diethoxy-pentyl ester 4

To an ice cooled solution of 2 (0.3 g, 1.7 mmol) and triethylamine (0.21 g, 2.0 mmol) in 3 ml CH₂Cl₂, methanesulfonyl chloride (0.21 g, 1.9 mmol) was added. After stirring for 1h at rt the mixture was washed with water and the solvent, dried on Na₂SO₄, evaporated in vacuo to give 4 (0.43g, 1.7 mmol, 100%), which was used for the subsequent reaction without further purification.

¹H NMR (CDCl₃): δ 4.47 (dt, 1H, J= 5.5 Hz, 2.5 Hz, C-1), 4.21 (dt, 2H, J=6.5 Hz, 2.7 Hz, C-5), 3.63 (m, 2H, CH₂ acetal), 3.48 (m, 2H, CH₂ acetal), 3.00 (s, 3H, OSO₂CH₃), 1.77 (m, 2H,

C-2), 1.63 (m, 2H, C-4), 1.47 (m, 2H, C-3), 1.20 (t, 6H, J=7.0 Hz, CH₃ acetal).

1-(5,5-diethoxypentyloxymethyl)-adamantane 5

5 NaH (60 % disp., 0.108 g, 2.7 mmol) was washed with pentane, and stirred with adamantanemethanol (0.3 g, 1.8 mmol) in 5 ml DMF, for 1h at rt yielding a suspension of the sodium salt. Compound 4 (0.4 g, 1.6 mmol) was added and the mixture was heated at 70°C for 4 h and stirred at rt
10 overnight. The mixture was treated with few drops of MeOH, poured into ice and extracted with diethyl ether (3x15 ml). The organic solvent, dried on Na₂SO₄, was evaporated and the residue purified by flash chromatography with petroleum ether 60-80/ethyl acetate 7:3 giving the desired compound 5 as a
15 viscous syrup.
5: yield 34%. ¹H NMR (CDCl₃): d 4.48 (t, 1H, J= 5.7 Hz, C-1 chain), 3.61 (dq, 2H, J=7.1 Hz, 2.3 Hz, CH₂ acetal), 3.49 (dq, 2H, J=7.1 Hz, 2.2 Hz, CH₂ acetal), 3.37 (t, 2H, J=6.5 Hz, C-5 chain), 2.94 (s, 2H, CH₂ adamant.), 1.94 (m, 20 3H, adamant.), 1.73-1.51 (m, 16H, C-2, C-4 chain, adamant.), 1.45-1.35 (m, 2H, C-3 chain), 1.19 (t, 6H, J=7.1 Hz, CH₃ acetal).

1-(5,5-Diethoxy-pentyloxy)-17-(dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene (O-(5,5-diethoxypentyl)-cholesterol) 6

25 NaH (60% disp., 0.12 g, 3 mmol) was washed with pentane and heated with cholesterol (1.16 g, 3 mmol) in DMF (6 mL) at 65-70°C during 45 min, yielding a suspension of the sodium
30 salt. 5,5-diethoxy-O-methanesulfonylpentanol 4 (0.508 g, 2 mmol) was added and the mixture was heated at 70-75°C during 20 h. The DMF was evaporated and the residue was extracted with ether and water. The ether extracts were dried (Na₂SO₄) and the residue after evaporation was purified by
35 flash chromatography (PE 60/80 - ethyl acetate 5/1) giving the product as a viscous syrup (0.63 g, 58%).

1H NMR (CDCl₃): d 5.31 (m, 1H, C-6 chol.), 4.45 (t, J=5.5 Hz, 1H, C-1 chain), 3.61 (dq, 2H, J=7.1 Hz, 2.4 Hz, CH₂ acetal), 3.45 (m, 4H, CH₂ acetal, C-5 chain), 3.10 (m, 1H, C-3 chol.), 2.34 (m, 1H, chol.), 2.16 (m, 1H, chol.), 2.07-1.70 (bm, 4H),
 5 1.70-0.75 (bm, 46H, chol., CH₃ acetal), 0.67 (s, 3H, CH₃ chol.).

5-[17-(dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta(a)phenanthren-3-yl-oxy]-pentanal (5-cholesterylpentanal) 7
 10 5-(adamantan-1-yl-methoxy)-pentanal 8

A mixture of the appropriate acetal 5, 6, (0.2 mmol) in 3 ml acetone and 1 ml 5% HCl was stirred at rt for 1h. Evaporation of the acetone, extraction of the residue with
 15 ether (3x7ml), drying on Na₂SO₄ and evaporation yielded the aldehyde (quant.) used for the next step without further purification.

7: yield 100%. 1H NMR (CDCl₃): d 9.77 (s, 1H, CHO), 5.33 (m, 1H, C-6 chol.), 3.46 (t, 2H, J=6.1 Hz, C-5 chain), 3.11 (m, 20 1H, C-3 chol.), 2.46 (dt, 2H, J=7.2 Hz, 1.4 Hz, C-2 chain) 2.34 (m, 1H, chol.), 2.16 (m, 1H, chol.), 2.05-1.75 (bm, 42H, C-3 C-4 chain, chol), 0.67 (s, 3H, CH₃ chol.).
 8: yield 100%. 1H NMR (CDCl₃): d 9.76 (t, 1H, J=1.7 Hz, CHO), 3.38 (t, 2H, J=6.2 Hz, C-5 chain), 2.94 (s, 2H, CH₂
 25 adamant.), 2.46 (dt, 2H, J=7.2 Hz, 1.7 Hz, C-2 chain), 1.95 (m, 3H, adamant.), 1.80-1.45 (m, 16H, C-3, C-4 chain, adamant.).

1-(5-[17-(dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta(a)phenanthren-3-yl-oxy]-pentyl)-2-hydroxymethyl-piperidine-3,4,5-triol
 30 (N-(5-cholesterylpentyl)-deoxynojirimycin 9

The aldehyde 7 (0.118 g, 0.25 mmol) was dissolved in a small amount of hot ethyl acetate, diluted with ethanol
 35 (about 4 ml) and added to a solution of DNM which was prepared by stirring DNJ.HCl (0.050 g, 0.2 mmol) and sodium

- acetate (0.020 g, 0.25 mmol) in methanol (0.4 ml) at rt for 1 h. NaCNBH₃ (0.016 g, 0.25 mmol) was added at 0°C and the suspension was stirred vigorously at rt during 18 h, and finally at 60°C for 1 h. After cooling to rt the mixture was acidified (pH < 1) with HCl (5%), stirred during 1 h and evaporated to dryness in vacuo. The remaining solids were suspended in a mixture of CH₂Cl₂ and methanol (1/1, 20 ml) and methanolic ammonia (20%, 2 ml) was added followed by silica (about 5 g). The mixture was (carefully) evaporated in vacuo to a free flowing solid, which was applied to a column of silica, pretreated with the eluent: CH₂Cl₂/MeOH/NH₃ in MeOH (20%) = 80/15/5. The column was eluted with 80/15/5, 75/20/5 and 70/25/5 mixtures of this eluent, resp., yielding pure 9 (solid, 0.081 g, 0.13 mmol, 65%) after evaporation.
- Hydrochloride: compound 9 (0.050 g) was dissolved in hot ethanol (20-30 ml) and treated with 3 drops of conc. HCl. Evaporation of the solvents yielded the hydrochloride as a crystalline solid (quant.), mp 235-238°C (sublimates from ca 190°C).
- ¹H NMR (D₂O): δ 5.36 (m, 1H, C-6 chol.), 4.10 (d, 1H, J= 12.6 Hz, C-6 DNM), 3.92 (d, 1H, J=12.6 Hz, C-6 DNM), 3.73 (m, 1H, C-2 DNJ), 3.61 (m, 1H, C-4 DNJ), 3.52 (t, 2H, J= 6.2 Hz, C-5 chain), 3.47 (dd, JC-1/C-1=12.1, JC-1/C-2=4.8, C-1 eq.DNM), 3.39 (m, 2H, C-3 DNM, C-1 chain), 3.29-3.05 (m, 3H, C-1 chain, C-3 chol., C-5 DNM), 3.00 (app.t, 1H, J= 11.6 Hz, C-1 ax. DNM), 2.34 (m, 1H, C-4 eq chol.), 2.15 (m, 1H, C-4 eq chol.), 2.03 (m, 1H, C-2 chol.), 1.97-0.80 (bm, 43H, C-2 C-4 C-3 chain, chol), 0.72 (s, 3H, CH₃ chol.). HRMS (FAB) obs mass 640.4979 (MNa⁺), calcd for C₃₈H₆₇NO₅Na 640.4917; obs mass 618.5086 (MH⁺), calcd for C₃₈H₆₈NO₅ 618.5097.

1-[5-adamantane-1-yl-methoxy)-pentyl]-2-hydroxymethyl-piperidine-3,4,5-triol (N-[5-adamantane-1-yl-methoxy)-pentyl]-deoxynojirimycin) 10

- A solution of deoxynojirimycin hydrochloride (0.030g, 0.15 mmol) and few µl of CH₃COOH in MeOH (2 ml) was added to

8 (0.056 g, 0.22 mmol) in MeOH (1 ml) at 0°C, followed by addition of NaCNBH₃ (0.014 g, 0.22 mmol). After stirring overnight at room temperature the reaction was concentrated, treated with 5% HCl (2 ml), stirred for 1h at rt and solid
5 Na₂CO₃ was added. The aqueous suspension was extracted with CH₂Cl₂ (3x7ml), the extracts combined, dried (Na₂SO₄) and evaporated in vacuo. The product was purified by silica gel flash chromatography (CH₂Cl₂/MeOH/8N NH₃ in MeOH 70:30:4), yielding pure 10 (0.030g, 0.08 mmol, 50%). The resulting oil
10 was dissolved in 5 ml MeOH and 1 ml 30% hydrochloric acid was added dropwise. The solvents and the excess of HCl were removed by coevaporation with methanol.
1H NMR (D₂O): d 4.11 (d, 1H, J=12.5 Hz, C-6 DNM), 3.98 (d, 1H, J=11.6 Hz, C-6 DNM), 3.82 (m, 1H, C-2 DNM), 3.69 (t, 1H, J=9.6 Hz, C-4 DNM), 3.62-3.45 (m, 4H, C-1 eq., C-3 DNM, C-5 chain), 3.36 (m, 1H, C-1 chain), 3.21 (m, 2H, C-5 DNM, C-1 chain), 3.08 (m, 3H, C-1 ax. DNM, CH₂ adamant.), 1.95 (m, 3H, adamant.), 1.90-1.56 (m, 10H, C-2, C-4 chain, adamant.), 1.52-1.30 (m, 8H, C-3 chain, adamant.). HRMS (FAB) obs mass
20 420.2745 (MNa⁺), calcd for C₂₂H₃₉NO₅Na 420.2726; obs mass 398.2905 (MH⁺), calcd for C₂₂H₄₀NO₅ 398.2906.

TABLE 1. OVERVIEW OF INHERITED SPHINGOLIPIDOSES IN MAN

DISEASE	DEFECTIVE ENZYME
5	Sphingolipidoses
	GM1 gangliosidosis β -galactosidase
	GM2 gangliosidosis/Tay Sachs hexosaminidase A
	GM2 gangliosidosis/Sandhoff hexosaminidase A and B
	Galactosialidosis protective protein
10	Ceramidetrihexoside α -galactosidase
	lipidosis/Fabry
	Metachromatic
	leukodystrophy arylsulfatase A
	Mucosulfatidosis
15	Glucosylceramide lipidosis/ multiple sulfatase deficiency
	Gaucher glucocerebrosidase
	Lipogranulomatosis/
	Farber ceramidase
	Sphingomyelin lipidosis/
20	Niemann-Pick A/B sphingomyelinase
	Galactosylceramide galactocerebrosidase
	lipidosis/Krabbe
	α -N-acetylgalacto-
	saminidase deficiency α -N-acetylgalactosaminidase
25	

TABLE 2. CHITOTRIOSIDASE ACTIVITY IN PLASMA AND LEUKOCYTES

Chitotriosidase activity in plasma samples from control subjects, asymptomatic Gaucher disease patients, and symptomatic Gaucher disease patients was determined as described in ref. 4. Chitotriosidase deficient individuals are not included in the table.

	PLASMA CHITOTRIOSIDASE (nmol/ml.h)		
35	CONTROLS	ASYMPTOMATICS	SYMPTOMATICS
	mean	98.4	16485
	n	5	30
	range	23.5-178.0	2949-55679
40			

TABLE 3. APPARENT K_i VALUES OF VARIOUS GLYCOSIDASES

Ki values were determined by variation of substrate concentration at fixed inhibitor concentration and assuming competitive inhibition and Michaelis-Menten kinetics. All constants are expressed in μM . (-) implies that no inhibition was noted at an inhibitor concentration of $100\mu\text{M}$. The structures of the tested inhibitors are depicted in Tables 6 and 7.

The activity of Ceredase towards 4MU-b-glucoside was determined in the presence of 0.25% (w/v) sodium taurocholate and 0.1% (v/v) Triton X-100 in citrate/phosphate buffer (pH 5.2). The activities of glucocerebrosidase and glucosylceramidase in membrane suspensions towards 4MU-b-glucoside were determined in citrate/phosphate buffer (pH 5.2). Conduritol B-epoxide was employed to discriminate between the two enzymes. The activity of lysosomal α -glucosidase towards 4MU- α -glucoside was determined in citrate/phosphate buffer at pH 4.0.

20	INHIBITOR	GLUCOSYL- CERAMIDASE	GLUCOCEREBROSIDASE CEREDASE MEMBRANES	A-GLUCO- SIDASE
25	DNJ	28.8	506	141
	PROPYLDNJ	0.123	546	332
	BUTYLDNJ	0.31	912	424
	PENTYLDNJ	0.038	249	8.5
	PENTANOYLDNJ	84	670	83
	P4	461	19.7	3.2
	P11	306	113	4.1
30	P16	39	11.6	0.44
	P9	-	51.6	-
	P13	-	11.2	-
	P21	0.0017	0.16	0.048
	P24	0.097	0.96	0.77
35	Km (mM)	3.28	3.25	1.45
				1.88

Note:

Apparent IC_{50} values of P21 and P24 for soluble glucocerebrosidase (Ceredase) were 0.2 and 0.8 μM .
 Apparent IC_{50} values of P21 and P24 for glucocerebrosidase in membrane suspension were 0.06 and 0.7 μM .

Apparent IC₅₀ values of P21 and P24 for glucosylceramidase were 1 nM and 0.1 μ M.

TABLE 4. IN VIVO INHIBITION BY DEOXYNOJIRIMYCIN ANALOGUES
 5 Melanoma cells were incubated with various concentrations of
 inhibitors to determine their IC₅₀ value (i.e. inhibitor
 concentration resulting in 50% inhibition). Activities of
 glucosylceramidase and glucocerebrosidase were determined as
 described in ref.17. NI=no significant inhibition detectable
 10 at 1 μ M inhibitor.

	INHIBITOR	IC ₅₀ (nM)	IC ₅₀ (nM)
		GLUCOSYLCERAMIDASE	GLUCOCEREBROSIDASE
15	DNJ	2000	NI
	PROPYLDNJ	650	NI
	BUTYLDNJ	200	NI
	PENTYLDNJ	150	NI
	PENTANOYLDNJ	30000	NI
	P4	200000	5000
20	P11	200000	8000
	P16	20000	NI
	P21	0.3	100
	P24	50	800
25			

TABLE 5. EFFECT OF DNJ ANALOGUES ON CULTURED MACROPHAGES

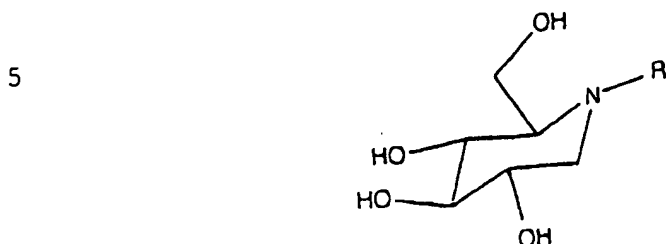
Human macrophages, obtained and cultured as described in ref. 4, were incubated with different concentrations butyl-deoxynojirimycin (BDNJ) or N-5-adamantane-1-yl-methoxy-pentyl)deoxynojirimycin (P21). After 4 days preincubation with inhibitor, glucosylceramidase and glucocerebrosidase activities were determined with C6NBD-glucosylceramide as substrate [17] and at the same time the released chitotriosidase in the medium was determined [4]. Enzyme activities and chitotriosidase secretion in the presence of DNJs are related to those in the absence of inhibitor (100%).

15	INHIBITOR		GLUCOSYL- CERAMIDASE ACTIVITY	GLUCOCERE- BOSIDASE ACTIVITY	CHITO- TRIOSIDASE SECRETION
			100	100	100
	NONE		100	100	100
20	B-DNJ (uM)	0.5	51	120	68
		5	12	112	49
		50	8	120	28
25	P21 (nM)	0.0025	90	120	105
		0.05	65	115	72
		1	40	130	64

Note:

Glucosylceramide synthase activity is not significantly inhibited at 5 uM B-DNJ or 1 nM P21. The presence of PDMP or PPMP, while potently inhibiting glucosylceramide synthase activity, does not result in reduced chitotriosidase secretion.

TABLE 6. N-Alkyl deoxynojirimycin derivatives



10

DNJ	$R = -H$
N-propyl DNJ	$R = -(CH_2)_2-CH_3$
N-butyl DNJ	$R = -(CH_2)_3-CH_3$
N-pentyl DNJ	$R = -(CH_2)_4-CH_3$
15 N-hexyl DNJ	$R = -(CH_2)_5-CH_3$
N-pentanoyl DNJ	$R = -CO-(CH_2)_3-CH_3$

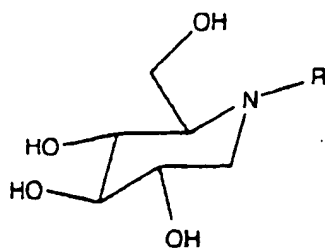
TABLE 7. N-Complex deoxynojirimycin derivatives (see next

20 page)

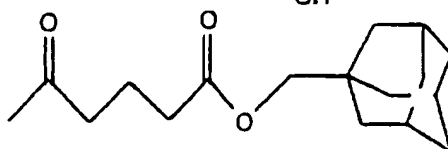
Names of the large apolar groups: 1. adamantanemethanol; 2. adamantanol; 3. 9-hydroxy-phenanthrene; 4. cholesterol; 5. β -cholestanol; 6. adamantanemethanol; 7. cholesterol.

25 In structure 1-5 the large apolar groups are linked to DNJ by a chain bearing two carbonyl groups. These two groups are replaced by methylene groups in structure 6 and 7.

5

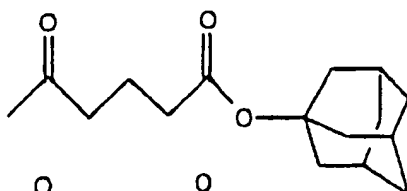


P4 R =



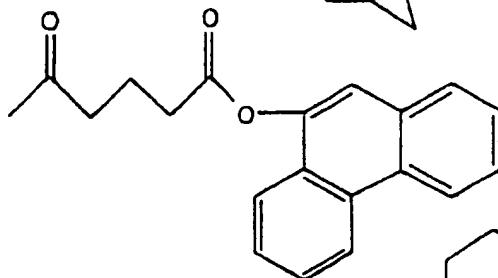
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P11 R =



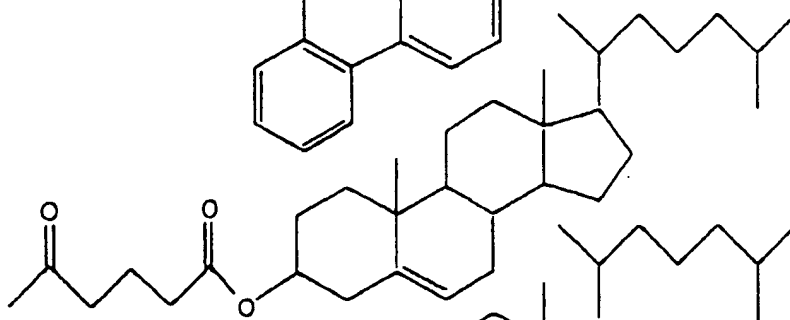
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P16 R =



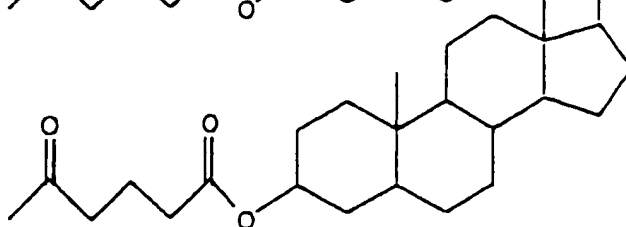
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P9 R =



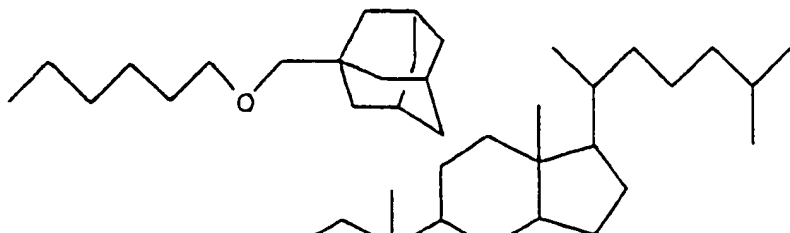
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P13 R =



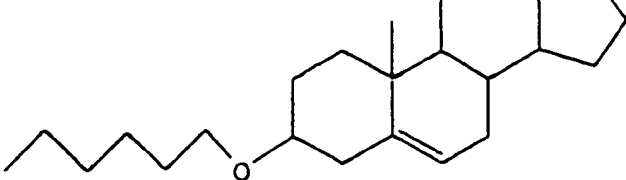
30

P21 R =



35

P24 R =



REFERENCES

1. Heller, R.A., Kronke, M. (1994) *J. Cell Biol.* **126**, 5-9.
Tumor necrosis factor receptor-mediated signalling pathways.
- 5 2. Hannun, Y.A. (1994) *J. Biol. Chem.* **269**, 3125-3128.
The sphingomyelin cyclin and the second messenger function of
ceramide.
3. Barranger, J.A., Ginns, E.I. (1989). Glucosylceramide
lipidoses: Gaucher's disease. In: *The Metabolic Basis of*
10 *Inherited Diseases*. C.R. Scriver, A.L. Beaudet, W.S. Sly & D.
Valle, editors; McGraw-Hill, Inc. New York, 1677-1698.
4. Hollak, C.E.M., van Weely, S., van Oers, M.H.J., Aerts,
J.M.F.G. (1994) *J. Clin. Invest.* **93**, 1288-1292. Marked
elevation of plasma chitotriosidase activity. A novel
15 hallmark of Gaucher disease.
5. Guo, Y., He, W., Boer, A.M., Wevers, R.A., de Bruyn,
A.M., Groener, J.E.M., Hollak, C.E.M., Aerts, J.M.F.G.,
Galjaard, H., van Diggelen, O.P. (1995) *J. Inher. Metab. Dis.*
18, 717-722. Elevated plasma chitotriosidase activity in
20 various lysosomal storage disorders.
6. Renkema, G.H., Boot, R.G., Muysers, A.O., Donker-
Koopman, W.E., Aerts, J.M.F.G. (1995) *J. Biol. Chem.* **270**,
2198-2202. Purification and characterization of human
chitotriosidase, a novel member of the chitinase family of
25 proteins.
7. Boot, R.G., Renkema, G.H., Strijland, A.H., van
Zonneveld, A.J., Aerts, J.M.F.G. (1995) *J. Biol. Chem.* **270**,
26252-26256. Cloning of a cDNA encoding chitotriosidase, a
human chitinase produced by macrophages.
- 30 8. Barton, N.W., Furbish, F.S., Murray, G.J., Garfield, M.,
Brady, R.O. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1913-1916.
Therapeutic response to intravenous infusions of glucocere-
brosidase in a patient with Gaucher disease.
9. Hollak, C.E.M., Aerts, J.M.F.G., Goudsmit, R., Phoa,
35 S.S.K.S., Ek, M., van Weely, S., von dem Borne, A.E.G.Kr.,

- van Oers, M.H.J. (1995) *Lancet* **345**, 1474-1478. Individualised low-dose alglucerase therapy for type 1 Gaucher's disease.
10. Grabowski, G.A., Barton, N.W., Pastores, G., Dambrosia, J.M., Banerjee, T.K., McKee, M.A., Parker, C., Schiffmann, R., Hill, S.C., Brady, R.O. (1995) *Ann. Int. Medicine* **122**, 33-39. Enzyme therapy in type 1 Gaucher disease: comparative efficacy of mannose-terminated glucocerebrosidase from natural and recombinant sources.
11. Aerts, J.M.F.G., Boot, R.G., Renkema, G.H., van Weely, S., Jones, S., Hollak, C.E.M., van Oers, M.H.J. (1995) *Sem. Hematol.* **32**, suppl. 1, 10-13. Molecular and biochemical abnormalities of Gaucher disease: chitotriosidase, a newly identified biochemical marker.
12. NIH Technology Assessment Panel on Gaucher Disease (1996) *JAMA* **275**, 548-553. Gaucher disease. Current issues in diagnosis and treatment.
13. Marshall, E. (1995) *Science* **269**, 1050-1055. Gene therapy's growing pains.
14. Abe, A., Radin, N.S., Shayman, J.A. (1996) *Biochim. Biophys. Acta* **1299**, 333-341. Induction of glucosylceramide synthase by synthase inhibitors and ceramide.
15. Platt, F.M., Neises, G.R., Dwek, R.A., Butters, T.D. (1994) *J. Biol. Chem.* **269**, 8362-8365. N-butyldeoxynojirimycin is a novel inhibitor of glycolipid biosynthesis.
16. Platt, F.M., Neises, G.R., Karlsson, G.B., Dwek, R.A., Butters, T.D. (1994) *J. Biol. Chem.* **269**, 27108-27114. N-butyldeoxygalactonojirimycin inhibits glycolipid biosynthesis but does not affect N-linked oligosaccharide processing.
17. van Weely, S., Brandsma, M., Strijland, A., Tager, J.M., Aerts, J.M.F.G. (1993) *Biochim. Biophys. Acta* **1181**, 53-62. Demonstration of the existence of a second, non-lysosomal glucocerebrosidase that is not deficient in Gaucher disease.
18. Overkleeft, H.S., van Wiltenburg, J., Pandit, U.K. (1994) *Tetrahedron* **34**, 4215-4224.

19. Baxter, E.W., Reitz, A.B. (1994) J. Org. Chem. **59**, 3175-3185.
20. Wanner, M.J., Koomen, G.J. (1995) J. Org. Chem. **60**, 5634-5637.

Claims

1. Deoxynojirimycin derivative containing a large hydrophobic moiety linked through a spacer to the nitrogen atom of deoxynojirimycin, and salts thereof.
2. Deoxynojirimycin derivative according to claim 1 wherein
5 the spacer comprises a polyalkylene chain of from 3 to 8 carbon atoms, preferably 3 to 6 carbon atoms, most preferably 5 carbon atoms.
3. Deoxynojirimycin derivative according to claim 1 wherein the spacer consists of a group having the structure $-(CH_2)_n-$
10 wherein n is an integer from 3 to 8, preferably 3 to 6, most preferably 5.
4. Deoxynojirimycin derivative according to claim 1 wherein the large hydrophobic moiety is derived from a compound selected from the group consisting of adamantanemethanol,
15 cholesterol, β -cholestanol, adamantanol and 9-hydroxyphenanthrene.
5. Deoxynojirimycin derivative according to claim 1 for use as an inhibitor of glucosylceramidase.
6. Deoxynojirimycin derivative according to claim 1 for use
20 in the treatment of a disease involving a ceramide-mediated signalling process, in particular by virtue of interference with ceramide-mediated signalling in inflammatory diseases.
7. Deoxynojirimycin derivative according to claim 1 for use in the treatment of a disease involving increased glucosyl-
25 ceramide levels in membranes containing glucosylceramidase, wherein said increased glucosylceramide levels are due to impaired glucosylceramide degradation in the lysosomes.
8. Deoxynojirimycin derivative according to claim 1 for use in the treatment of a lysosomal lipid storage disorder.
- 30 9. Deoxynojirimycin derivative according to claim 1 for use in the treatment of Gaucher disease.

10. Pharmaceutical composition comprising a deoxynojirimycin derivative according to any one of claims 1 to 4 and a pharmaceutically acceptable carrier.
11. Use of a deoxynojirimycin derivative according to any one of claims 1 to 4 as an inhibitor of glucosylceramidase.
12. Use of a glucosylceramidase inhibitor for preparing a pharmaceutical composition for use in the treatment of Gaucher disease or other diseases, in particular inflammatory diseases, in which a ceramide-mediated signalling process is involved.
13. Use of a deoxynojirimycin derivative according to any one of claims 1 to 4 for preparing a pharmaceutical composition for use in the treatment of Gaucher disease or other diseases, in particular inflammatory diseases, in which a ceramide-mediated signalling process is involved.
14. Use of a deoxynojirimycin derivative according to any one of claims 1 to 4 for preparing a pharmaceutical composition for use in the treatment of a lysosomal lipid storage disorder.
15. A method of treatment of an individual suffering from Gaucher disease, comprising administering to said individual an effective amount of a glucosylceramidase inhibitor, optionally in combination with an effective amount of native or recombinant, modified or unmodified glucocerebrosidase.
16. A method of treatment of an individual suffering from Gaucher disease, comprising administering to said individual an effective amount of a deoxynojirimycin derivative according to any one of claims 1 to 4, optionally in combination with an effective amount of native or recombinant, modified or unmodified glucocerebrosidase.

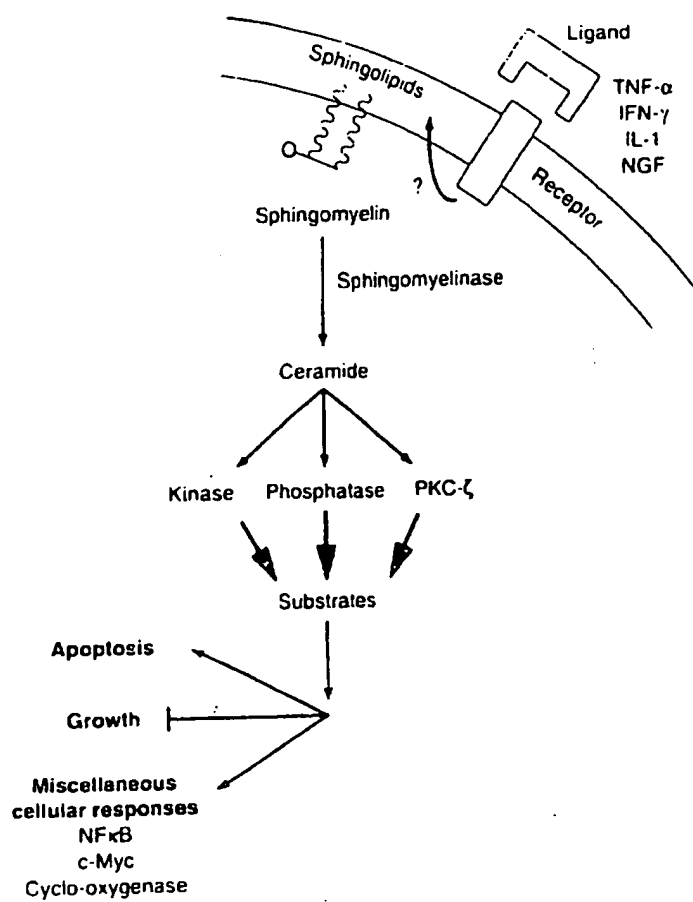


FIG.1.

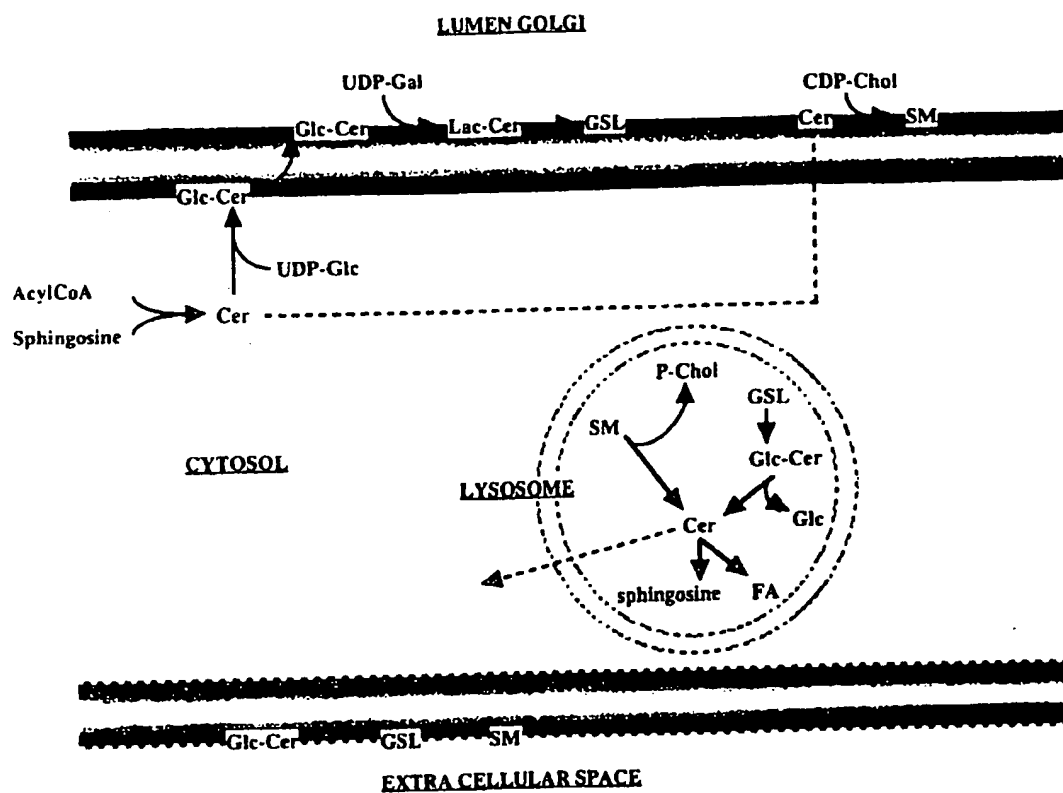


FIG.2.

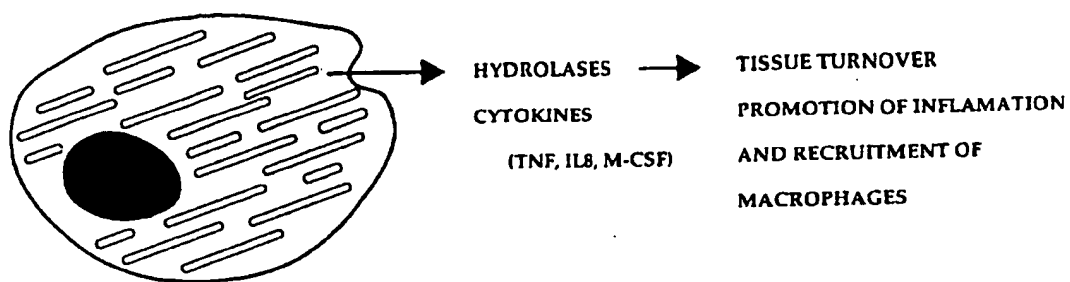


FIG.3.

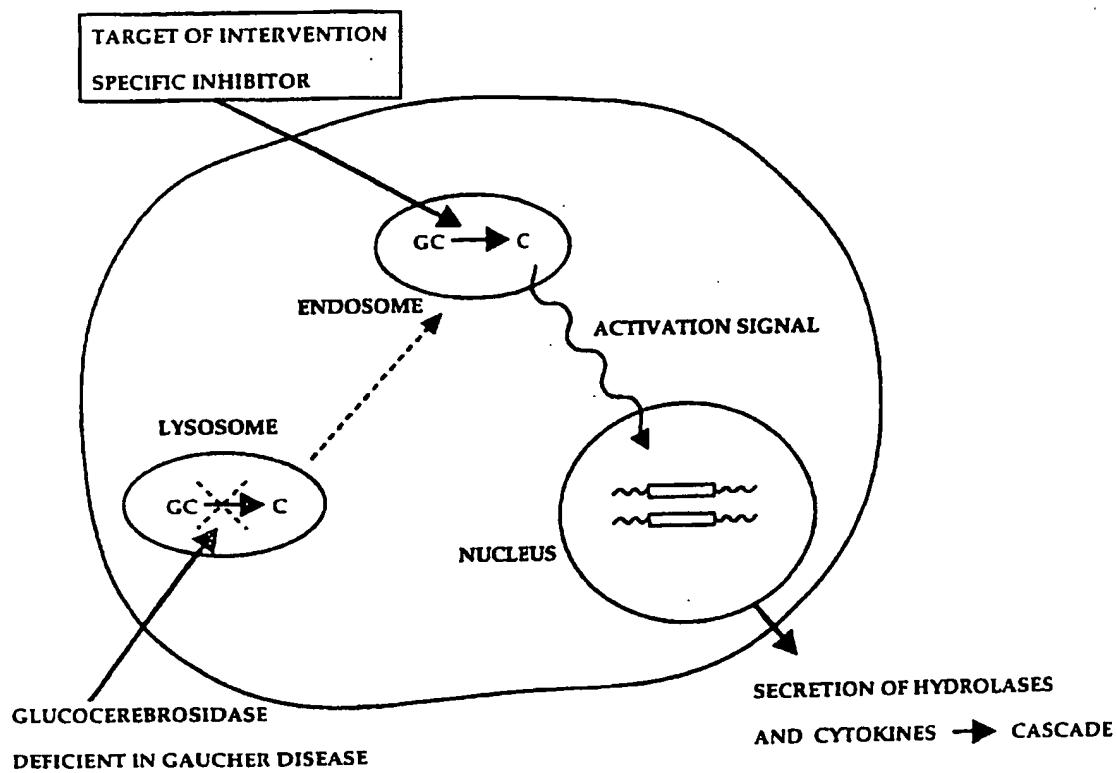


FIG.4.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 97/00411

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/445 C07D211/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 22975 A (G.D. SEARLE & CO.) 31 August 1995 see claims 1-7 see examples 1,11,13,32,43 ---	1-3,5-10
X	EP 0 477 160 A (MONSANTO CO ET AL) 25 March 1992 see claims 20-45 see page 8, line 33 - page 9, line 22 ---	1-3,5-10 11,13,14
Y	---	
	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

S document member of the same patent family

Date of the actual completion of the international search

14 October 1997

Date of mailing of the international search report

29.10.1997

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Siatou, E

INTERNATIONAL SEARCH REPORT

Inter. Appl. No.

PCT/NL 97/00411

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	F. M. PLATT ET AL: "N-Butyldeoxynojirimycin Is a Novel Inhibitor of Glycolipid Biosynthesis" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 11, 18 March 1994, USA, pages 8362-8365, XP000615445 cited in the application	12
Y	see abstract see page 8364, right-hand column, line 6 - page 8365, left-hand column, line 45	11,13,14
X	EP 0 350 012 A (MEIJI SEIKA KAISHA LTD ET AL) 10 January 1990 see claims 1-4 see page 4, line 8 - line 21	1-3,5-10
X	PATENT ABSTRACTS OF JAPAN vol. 15, no. 92 (C-0811), 6 March 1991 & JP 02 306962 A (MEIJI SEIKA KAISHA LTD), 20 December 1990, see abstract	1-3,5-10
X	EP 0 193 770 A (BAYER AG) 10 September 1986 see claims 1-5,11-14	1-3,5-10
X	EP 0 034 784 A (BAYER AG) 2 September 1981 see claims 1,2,5-9 see page 3, line 1 - line 6	1-3,5-10
X	EP 0 022 192 A (BAYER AG) 14 January 1981 see claims 1,2,5-8 see page 1, line 6 - page 2, line 2	1-3,5-10
X	DE 30 24 901 A (BAYER AG) 28 January 1982 see claims 1-3 see examples 2,6,7	1-3,5-9
A	WO 94 13311 A (ENZON INC.) 23 June 1994 see claims 1-31	1-10, 12-14
A	E. BEUTLER: "Gaucher Disease: New Molecular Approaches to Diagnosis and Treatment" SCIENCE, vol. 256, 8 May 1992, USA, pages 794-799, XP000371643 see the whole document	1-10, 12-14

INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 97/00411

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	C. E. HOLLAK ET AL: "Marked Elevation of Plasma Chitotriosidase Activity. A Novel Hallmark of Gaucher Disease" JOURNAL OF CLINICAL INVESTIGATION, vol. 93, March 1994, USA, pages 1288-1292, XP000609695 cited in the application see abstract	1-10, 12-14
A	--- J. M. F. G. AERTS ET AL: "Molecular and Biochemical Abnormalities of Gaucher Disease: Chitotriosidase, a Newly Identified Biochemical Marker" SEMINARS IN HEMATOLOGY, vol. 32, no. 3, Sup, July 1995, pages 10-13, XP000609802 cited in the application see abstract -----	1-10, 12-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 97/00411

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 15-16
because they relate to subject matter not required to be searched by this Authority, namely:
Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
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Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/NL 97/00411

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9522975 A	31-08-95	AU 1876095 A US 5622972 A	11-09-95 22-04-97
EP 0477160 A	25-03-92	AT 137266 T CA 2051855 A DE 69119010 D ES 2087277 T JP 4342567 A US 5610039 A US 5602013 A	15-05-96 21-03-92 30-05-96 16-07-96 30-11-92 11-03-97 11-02-97
EP 0350012 A	10-01-90	JP 2131425 A	21-05-90
EP 0193770 A	10-09-86	DE 3507019 A JP 61200967 A	28-08-86 05-09-86
EP 0034784 A	02-09-81	DE 3007078 A AT 8623 T AU 538463 B AU 6748081 A CA 1160634 A DK 84181 A JP 1384503 C JP 56133267 A JP 61052147 B JP 1679326 C JP 3042243 B JP 60166616 A JP 60166663 A SU 1014471 A US 4407809 A ZA 8101252 A	10-09-81 15-08-84 16-08-84 03-09-81 17-01-84 27-08-81 26-06-87 19-10-81 12-11-86 13-07-92 26-06-91 29-08-85 29-08-85 23-04-83 04-10-83 31-03-82
EP 0022192 A	14-01-81	DE 2925943 A AT 3421 T AU 5933080 A CA 1138879 A DK 274980 A JP 1303453 C JP 56007763 A	29-01-81 15-06-83 08-01-81 04-01-83 28-12-80 28-02-86 27-01-81

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/NL 97/00411

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0022192 A		JP 60026388 B US 4312872 A ZA 8003818 A	24-06-85 26-01-82 29-07-81
DE 3024901 A	28-01-82	NONE	
WO 9413311 A	23-06-94	AU 5743994 A EP 0675727 A JP 8507677 T US 5620884 A	04-07-94 11-10-95 20-08-96 15-04-97

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 31/445 // 31/70		A1	(11) International Publication Number: WO 98/30219						
			(43) International Publication Date: 16 July 1998 (16.07.98)						
(21) International Application Number: PCT/US98/00031		Glycobiology Institute, Dept. of Biochemistry, South Parks Road, Oxford OX1 3QU (GB).							
(22) International Filing Date: 13 January 1998 (13.01.98)		(74) Agents: MEYER, Scott, J. et al.; G.D. Searle & Co., Corporate Patent Dept., P.O. Box 5110, Chicago, IL 60680-5110 (US).							
(30) Priority Data: 08/782,321 13 January 1997 (13.01.97) US		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).							
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/782,321 (CON) Filed on 13 January 1997 (13.01.97)		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.							
(71) Applicant (for all designated States except US): MONSANTO COMPANY [US/US]; 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US).									
(72) Inventors; and (75) Inventors/Applicants (for US only): PLATT, Frances, M. [GB/GB]; University of Oxford, Glycobiology Institute, Dept. of Biochemistry, South Parks Road, Oxford OX1 3QU (GB). NEISES, Gabrielle, R. [US/US]; 13939 Meursault, Chesterfield, MO 63017 (US). DWEK, Raymond, A. [GB/GB]; University of Oxford, Glycobiology Institute, Dept. of Biochemistry, South Parks Road, Oxford OX1 3QU (GB). BUTTERS, Terry, D. [GB/GB]; University of Oxford,									
(54) Title: METHOD FOR TREATMENT OF CNS-INVOLVED LYSOSOMAL STORAGE DISEASES									
(57) Abstract									
<p>A method is disclosed for the <i>in vivo</i> treatment of patients having a lysosomal storage disease with a significant central nervous system (CNS) involvement. Said method comprises administration to said patient a small but storage-inhibitory effective amount of an N-alkyl derivative of a 1,5-iminosugar in which said alkyl group contains from about 2 to about 8 carbon atoms and said 1,5-iminosugar is 1,5-dideoxy-1,5-imino-D-glucitol, or 1,5-dideoxy-1,5-imino-D-galactitol, or an O-acylated pro-drug of said 1,5-iminosugar. In an illustrative example, CNS storage of GM2 ganglioside is inhibited in Tay-Sachs mice by administration of 1,5-(butylimino)-1,5-dideoxy-D-glucitol.</p>									
<table border="1"> <caption>GM2 (arbitrary units) by Treatment</caption> <thead> <tr> <th>Treatment</th> <th>GM2 (arbitrary units)</th> </tr> </thead> <tbody> <tr> <td>Untreated</td> <td>~27</td> </tr> <tr> <td>+ NB-DNJ</td> <td>~12</td> </tr> </tbody> </table>				Treatment	GM2 (arbitrary units)	Untreated	~27	+ NB-DNJ	~12
Treatment	GM2 (arbitrary units)								
Untreated	~27								
+ NB-DNJ	~12								

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EE	Estonia						

METHOD FOR TREATMENT OF
CNS-INVOLVED LYSOSOMAL STORAGE DISEASES

CROSS-REFERENCE TO RELATED APPLICATIONS

This is a continuation-in-part of application Ser. No.
08/393,640, filed February 24, 1995,

- which is a continuation of application Ser. No.
08/061,645, filed May 13, 1993, now U.S. Patent
5,399,567,
- and is a continuation-in-part of application Ser. No.
08/588,027, filed January 17, 1996,
- which is a division of application Ser. No. 08/396,989,
filed March 1, 1995,
- which is a division of application Ser. No. 08/102,654,
filed August 5, 1993,
- which is a continuation-in-part of said application
Ser. No. 08/061,645.

BACKGROUND AND FIELD OF THE INVENTION

This invention relates to a method for the treatment of lysosomal storage diseases that have a significant central nervous system (CNS) involvement. These diseases are caused by genetic mutations which result in the absence or deficiency of lysosomal enzymes. They include, for example, Tay-Sachs disease, Sandhoff disease, GM1 gangliosidosis and Fabry disease.

A list of references indicated by numerals in parentheses is appended at the end.

TAY-SACHS DISEASE:

This is a fatal hereditary disorder of lipid metabolism characterized especially in CNS tissue due to deficiency of the A (acidic) isozyme of β -hexosaminidase. Mutations in the *HEXA* gene, which encodes the α subunit of β -hexosaminidase, cause the A isozyme deficiency.

Tay-Sachs [disease] is a prototype of a group of disorders, the GM2 gangliosidoses, characterized by defective GM2 ganglioside degradation. The GM2 ganglioside (monosialylated ganglioside 2) accumulates in the neurons beginning already in fetal life.

SANDHOFF DISEASE:

Sandhoff disease results from a deficiency of both the A and B (basic) isozymes of β -hexosaminidase. Mutations in the *HEXB* gene, which encodes the β subunit of β -hexosaminidase, cause the B isozyme deficiency.

GM1 GANGLIOSIDOSIS:

GM1 gangliosidosis is caused by a deficiency of β -galactosidase, which results in lysosomal storage of GM1 ganglioside (monosialylated ganglioside 1).

FABRY DISEASE:

Fabry disease is caused by a deficiency of α -galactosidase which results in lysosomal storage of a ceramide trihexoside.

Glycosphingolipid (GSL) storage diseases are a group of human autosomal recessive disorders (except Fabry disease which is X-linked), each of which exhibits a characteristic pathology (1). They result from the inheritance of defects in genes encoding the catabolic enzymes required for the complete breakdown of GSLs within the lysosomes.

There presently is no effective therapy for Tay-Sachs disease or other lysosomal storage diseases with CNS involvement. Proposed strategies for the treatment of these debilitating and often fatal diseases include enzyme replacement therapy, gene therapy, substrate deprivation, allogenic bone marrow transplantation and palliative measures (2). Of these, symptomatic management is the only approach for treating most of these disorders, although transplantation techniques have been applied to some of these diseases.

Currently, only the non-neuronopathic form of Gaucher disease (type 1), a condition characterized by glucocerebrosidase deficiency, which occurs at high frequency in Ashkenazi Jews, has been successfully treated using enzyme replacement therapy (3,4). However, skeletal abnormalities associated with the disease respond slowly to this treatment (4) and the rare type 2 (acute neuronopathic; infantile) and rare type 3 (chronic; juvenile) are refractory to therapy.

Accordingly, new therapeutic treatment for lysosomal storage diseases which have significant CNS involvement (neuronopathic) are urgently needed.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, a method is provided for the in vivo treatment of patients having a lysosomal storage disease with a significant CNS involvement.

Said method comprises administration to said patient of a small but storage-inhibitory effective amount of an N-alkyl derivative of a 1,5-iminosugar in which said alkyl group contains from about 2 to about 8 carbon atoms and said 1,5-iminosugar is 1,5-dideoxy-1,5-imino-D-glucitol, or 1,5-dideoxy-1,5-imino-D-galactitol, or an O-acylated pro-drug of said 1,5-iminosugar.

Preferred 1,5-iminosugars are:

1,5-(Butylimino)-1,5-dideoxy-D-glucitol, which is also known as N-butyl deoxynojirimycin or by the abbreviated designation N-butyl DNJ;

1,5-(Butylimino)-1,5-dideoxy-D-galactitol, which is also known as N-butyl deoxygalactonojirimycin or by the abbreviated designation N-butyl DGJ;

1,5-(Butylimino)-1,5-dideoxy-D-glucitol, tetrabutyrates; and

1,5-(Butylimino)-1,5-dideoxy-D-galactitol, tetrabutyrates.

The method of the invention is illustrated in detail herein with the preferred compound, N-butyl DNJ. As described in detail herein, a mouse model of Tay-Sachs disease (9) is used to illustrate the in vivo effect of the 1,5-iminosugars for treatment of lysosomal storage diseases having a significant CNS involvement. Using this mouse model, it was demonstrated with the illustrative

N-butyl DNJ that this agent unexpectedly was able to cross the blood:brain barrier to an extent which inhibited CNS storage of GM2 ganglioside compared to the untreated control mice which exhibit progressive storage of that ganglioside.

DETAILED DESCRIPTION OF THE INVENTION

While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the invention, it is believed that the invention will be better understood from the following illustrative detailed description taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows thin layer chromatography (TLC) analysis of GM2 ganglioside storage in the Tay-Sachs mouse in the presence or absence of N-butyl deoxynojirimycin (NB-DNJ). Mice were treated with NB-DNJ from 4 weeks of age up to twelve weeks and their GSL (glycosphingolipid) profiles compared at 4, 8 and 12 weeks relative to the untreated age matched controls. Each lane on the TLC plate represents the base-resistant GSLs derived from the whole brain of an individual mouse. The data are representative of studies carried out on five mice at each time point. The migration position of an authentic GM2 standard is indicated with an arrow.

FIG. 2, in two parts, A and B, shows prevention of GM2 storage in 12 week old mice. To demonstrate the variation in GM2 storage in mice treated with NB-DNJ, a group of three untreated and three NB-DNJ treated mice were compared at 12 weeks of age.

FIG. 2A: TLC profiles on total brain GSLs for three untreated mice (-) and three NB-DNJ treated mice (+).

FIG. 2B: Scanning densitometry on the GM2 species from FIG. 2A expressed in arbitrary units. The mean values +/- standard deviation are shown.

FIG. 3, in four parts, A-D, shows GM2 storage in the ventromedial hypothalamus of untreated and NB-DNJ treated mice (12 weeks of age). Frozen sections were stained with periodic acid-Schiff (PAS) to allow the visualization of GM2 storing neurons.

FIG. 3A: Untreated mouse, 10x magnification;

FIG. 3B: NB-DNJ treated mouse, 10x magnification;

FIG. 3C: Untreated mouse, 25x magnification; and

FIG. 3D: NB-DNJ treated mouse, 25x magnification.

The sections were selected to ensure that sections from the two animals were comparable in terms of spatial orientation within the brain. The images shown are representative of data derived from four different pairs of mice. The reduction in PAS staining in NB-DNJ treated mice was also observed in other storage regions of the brain.

FIG. 4, in four parts, A-D, shows electron microscopy of brains from untreated and NB-DNJ treated mice.

FIG. 4A: GM2 storage neuron from an untreated mouse brain;

FIG. 4B: GM2 storage neuron from an NB-DNJ treated mouse brain (the scale bar for A and B represents 1 μ m;

FIG. 4C: MCBs (membranous cytoplasmic bodies) from an untreated mouse brain;

FIG. 4D: MCBs from an NB-DNJ treated mouse brain (the scale bar for C and D represents 0.1 μ m).

The data shown are representative on the basis of analyzing multiple sections for multiple storage regions of the brain from two untreated and two NB-DNJ treated animals, with the analysis carried out by two independent groups.

FIG. 5 is a schematic flow chart which shows glycolipid catabolism and the relationship of lysosomal storage diseases to the absence or deficiency of relevant enzyme to degrade a given ganglioside or globoside. Of the lysosomal storage diseases that have a significant CNS involvement:

- * GM1 gangliosidosis is shown to be caused by a deficiency in β -galactosidase;
- * Tay-Sachs disease, which is a GM2 gangliosidosis, is shown to be caused by a deficiency of β -hexosaminidase A (acidic isozyme);
- * Sandhoff disease is shown to be caused by a deficiency of β -hexosaminidase A & B (acidic and basic isozymes); and
- * Fabry disease is shown to be caused by a deficiency in α -galactosidase.

In a different step of the GlcCer pathway, Gaucher disease is shown to be caused by a deficiency in β -glucocerebrosidase. Three diseases shown at the bottom of FIG. 5, namely metachromatic leukodystrophy (MLD), Krabbe disease and Niemann-Pick disease, involve different metabolic pathways, the GalCer pathway and sphingomyelinase (SM) pathway.

The mouse model of Tay-Sachs disease (9) is a useful model for demonstrating the in vivo effectiveness of the 1,5-iminosugars for the treatment of lysosomal storage diseases having a significant CNS involvement since this model has all the hallmarks of Tay-Sachs disease.

Tay-Sachs disease results from mutations in the *HEXA* gene, which encodes the α subunit of β -hexosaminidase, leading to a deficiency in the A isoenzyme. The A isoenzyme is responsible for the degradation of GM2 ganglioside. When this enzyme is deficient in humans, GM2 ganglioside accumulates progressively and leads to severe neurological degeneration (10).

In the mouse model of Tay-Sachs disease (generated by the targeted disruption of the *Hexa* gene), the mice store GM2 ganglioside in a progressive fashion, but the levels never exceed the threshold required to elicit neurodegeneration (9).

This is because in the mouse (but not human) a sialidase is sufficiently abundant that it can convert GM2 to GA2 (asialo ganglioside 2), which can then be catabolized by the hexosaminidase B isoenzyme (11). This model therefore has all the hallmarks of Tay-Sachs disease, in that it stores GM2 ganglioside in the CNS, but it never develops the neurological symptoms characteristic of the human disease (9,11,12).

In order to thus further illustrate the invention, the following detailed examples were carried out although it will be understood that the invention is not limited to these specific examples or the details described therein.

EXAMPLE I

Tay-Sachs mice were reared on standard mouse chow up to the age of weaning (4 weeks post-partum) when they were placed on a powdered mouse chow diet containing NB-DNJ as follows:

Mice were fed on a diet of powdered mouse chow (expanded Rat and Mouse Chow 1, ground, SDS Ltd., Witham, Essex, UK) containing NB-DNJ from weaning (4 weeks). The diet and compound (both dry solids) were mixed thoroughly before use, stored at room temperature and used within seven days of mixing. Water was available to the mice *ad lib*. The mice were housed under standard non-sterile conditions. The mice were given a dosing regime of 4800mg/kg/day of NB-DNJ which gave serum levels of approximately 50 μ M.

Similar serum levels (steady state trough level of approximately 20 μ M) were achieved in humans during the evaluation of this compound as an anti-viral agent when patients were treated with 43 mg/kg/day (12).

The pharmacokinetics of NB-DNJ are two orders of magnitude poorer in mouse relative to human, thereby necessitating high dosing regimes in the mouse in order to achieve serum levels in the predicted therapeutic range for the GSL storage disorders of 5-50 μ M (5-8).

EXAMPLE II

The effects that drug administration had on GM2 storage in the Tay-Sachs mouse were determined at various ages by extracting total brain lipids, separating the base resistant GSL fraction by TLC (FIG. 1), and identifying the GM2 species on the basis of co-migration with an authentic GM2 standard. The following procedure was employed:

The animals were anesthetized, perfused with phosphate buffered saline, pH 7.2, and the intact brain removed. The brain tissue was manually homogenized in water, freeze-dried and extracted twice with chloroform:methanol 2:1 (v/v) for two hours at room temperature and overnight at 4°C.

A volume of the solvent extract equivalent to 5 mg dry weight for each brain was dried under nitrogen, taken up in 500µl chloroform:methanol (1:1v/v), 83µl of 0.35M NaOH in 96% methanol added and incubated at room temperature for 90 mins. The samples were partitioned by adding 83µl H₂O:methanol (9:1 v/v), 166.5µl H₂O and 416µl chloroform, spun in a microfuge for 1 min and the upper phase retained. The lower phase was washed twice in Folch theoretical upper phase (chloroform:methanol:water, 1:10:10 v/v/v) and the upper phases retained and pooled with the original upper phase.

The samples were partially dried under N₂ to remove the solvent and the residual aqueous sample made up to 1ml with H₂O and dialyzed overnight in 2 liters of water to desalt. The samples were freeze-dried, extracted with 500µl chloroform:methanol 2:1 v/v, spun at 13000rpm for 2 mins and the supernatant retained, dried under N₂, resuspended in 10µl chloroform:methanol:0.22% calcium chloride (60:35:8 v/v/v) and separated by TLC (Silica gel

60 plates, Merck, BDH, Poole, Dorset, UK) in chloroform:methanol:calcium chloride (60:35:0.22%), sprayed with orcinol and visualized by heating to 80°C for 10 mins.

By four weeks of age a storage band corresponding to GM2 was detectable in the untreated mice, in agreement with previously published reports on this mouse model (9). As expected on the basis of published studies (9), the accumulation of GM2 in the untreated mice progressively increased with increasing age of the mice (FIG. 1).

However, in the NB-DNJ treated mice by eight weeks of age (four weeks untreated from birth to weaning, 4 weeks NB-DNJ treated post-weaning) there was an unexpected reduction in the intensity of the GM2 ganglioside band, relative to the untreated age matched controls, indicating that reduced levels of storage were occurring in the presence of the drug. The mice were followed for twelve weeks and there was a consistent reduction in stored GM2 ganglioside in all animals from the NB-DNJ treated group, irrespective of their age (FIG. 1).

To examine the generality of these data a group of three untreated and three NB-DNJ treated mice were evaluated at 12 weeks (FIG. 2A). In all cases, the intensity of the GM2 band was significantly reduced in the NB-DNJ treated animals, relative to the untreated age matched controls. When scanning densitometry was performed on the TLC profiles it was found that there was an approximately 50% reduction in GM2 ganglioside in the treated mouse brains relative to the untreated controls (FIG. 2B).

EXAMPLE III

The neurons within the Tay-Sachs mouse brains which are responsible for the GM2 storage observed in whole brain lipid extracts are confined to certain specific regions of the brain (12). We therefore carried out cytochemical analysis on tissue sections from untreated mice and mice treated for 16 weeks with NB-DNJ using periodic acid-Schiff (PAS) staining to detect the stored ganglioside within the storage neurons (9), as follows:

Mice were anesthetized, perfused with phosphate buffer pH 7.4 containing 4% paraformaldehyde and the brain dissected and retained in fixative overnight prior to cryopreservation and sectioning. Frozen brain sections (7 micron) were warmed to room temperature, stained with periodic acid-Schiff (PAS) according to the manufacturer's instructions (Sigma, Poole Dorset UK), counter-stained with Ehrlich's hematoxylin and mounted in DPX (BDH).

It has previously been demonstrated in these untreated mice that the distribution of neurons staining with PAS is coincident with neurons which immunostain with an antibody specific for GM2 ganglioside (9).

In storage regions of the brain, such as the ventromedial hypothalamic nucleus, the NB-DNJ treated mice had fewer PAS positive neurons and the intensity of staining in each neuron was reduced (FIGS. 3B and 3D), relative to the untreated age matched control's brain sections, which exhibited extensive storage (FIGS. 3A and 3C).

The status of the GM2 storage in individual neurons from treated and untreated mouse brains was examined by electron microscopy (EM) as follows:

The mice were anesthetized and perfusion fixed with 2% paraformaldehyde, 2% glutaraldehyde mix in PBS. The brain was dissected and fixed in the same fixative overnight at 4°C. The brain was trimmed and 100µm sections cut on a vibrotome, the sections washed three times in 0.1M phosphate buffer and stained with osmium tetroxide (1% in 0.1M phosphate) for 35 mins. The sections were dehydrated through an ethanol series, treated with propylene oxide (2 x 15 mins) and placed in Durcupan resin overnight at room temperature, transferred to glass slides and placed at 60°C for 48 hours.

Storage areas of the brain were selected microscopically, cut out of the thick section with a scalpel blade and glued with Super Glue Loctite, Quick Tite, (Loctite Corp., Rock Hill, CT) onto an Embed 800 stub (Electron Microscopy Sciences, Fort Washington, PA). Sections were stained with uranyl acetate/lead citrate and observed with a Hitachi 600 microscope at 75 kv.

In the storage neurons from untreated Tay-Sachs mouse brains there were prominent regions of the cytoplasm containing large numbers of membranous cytoplasmic bodies (MCBs) containing the stored lipid product (FIG. 4A). In contrast, in the NB-DNJ treated mice it proved difficult to find storage neurons. However, when storage cells could be located they contained MCBs with greatly reduced electron dense contents (FIG. 4B).

Furthermore, the extensive storage observed within storage neurons from untreated mice resulted in the organelles with the highest degree of storage being difficult to section, with the storage product frequently detaching partially from the surrounding membrane (FIG. 4A). In the NB-DNJ treated mouse brains the storage within neurons was always markedly reduced, relative to the untreated controls, and as a result no sectioning artifact was observed (FIG. 4B). This was consistently observed by two independent electron microscopy groups studying independent

material derived from these mice. One representative set of data is shown in FIG. 4.

The EM data are in keeping with the cytochemical staining which indicated that there were fewer storage neurons in the brains of treated mice and that storage cells in the treated animals had reduced levels of GM2 storage, relative to the untreated controls. When the morphology of individual MCBs from untreated and NB-DNJ treated mice were compared under high magnification by EM there was a profound difference in their morphology.

The NB-DNJ treated mice had MCBs which contained less electron dense storage lipid (FIG. 4D) but also did not have the prominent concentrically arranged lamellae characteristic of the MCB in neurons from untreated mice (FIG. 4C). Instead, they exhibited a diffuse pattern of storage with membrane-like structures only clearly discernible in the periphery of the organelle (FIG. 4D). Taken together with the cytochemical data, this demonstrates that NB-DNJ prevents lysosomal storage and the extent of storage per cell and per MCB is dramatically reduced, in keeping with the biochemical data on whole brain GSLs (FIGS. 1 and 2).

The data outlined herein demonstrate that oral treatment of mice with NB-DNJ is well tolerated and that it results in the inhibition of GSL biosynthesis. Furthermore, in the Tay-Sachs mouse, which exhibits progressive CNS storage of GM2 ganglioside, we have been able to prevent storage, as a consequence of reducing GSL biosynthesis. This indicates that NB-DNJ can cross the blood:brain barrier to an extent which can prevent storage.

This therefore indicates that substrate deprivation resulting from NB-DNJ administration is a rational strategy for the therapy of the human GSL lysosomal storage diseases. It has been shown in vitro that NB-DNJ specifically inhibits the first step in GSL

biosynthesis, the glucosyl-transferase catalyzed biosynthesis of GlcCer (5-7).

As several of the human glycosphingolipid (GSL) storage diseases involve the storage of GlcCer-based GSLs, this therapeutic strategy can be applied to all of these disease states, irrespective of the specific storage product. This would include Gaucher (types 1, 2 and 3), Fabry disease, Tay-Sachs disease, Sandhoff disease, GM1 gangliosidosis, and fucosidosis.

The current application of enzyme replacement to Gaucher disease is limited by the fact that the enzyme cannot cross the blood:brain barrier and hence this therapy is only efficacious in type 1 disease where there is no neuropathology involved. Our finding that GSL depletion can be achieved in the central nervous system is therefore of major significance as it means that all the GSL storage diseases could be treated with NB-DNJ, as many of them involve neuropathology in the CNS.

NB-DNJ does not appear to inhibit galactosyltransferase which initiates the biosynthesis of the pathway that results in the formation of GalCer and sulphatide. Therefore, it is not believed that NB-DNJ would show efficacy against Krabbe disease and metachromatic leukodystrophy (MLD). These diseases involve the storage of GalCer and sulphatide, respectively, as shown at the bottom of FIG. 5. This can be advantageous to the invention as the formation of GalCer and sulphatide, which are important constituents of myelin, would not be affected by the treatment. Hence, myelination and myelin stability would not be impaired.

EXAMPLE IV

When any of the following compounds are substituted for an equivalent amount of 1,5-(Butylimino)-1,5-dideoxy-D-glucitol in the above Examples I, II and III, substantially similar inhibitory results are obtained:

- A) 1,5-(Hexylimino)-1,5-dideoxy-D-glucitol;
- B) 1,5-(Butylimino)-1,5-dideoxy-D-galactitol;
- C) 1,5-(Hexylimino)-1,5-dideoxy-D-galactitol;
- D) 1,5-(Butylimino)-1,5-dideoxy-D-glucitol, tetrabutyrates;
- E) 1,5-(Hexylimino)-1,5-dideoxy-D-glucitol, tetraacetate.

Compounds D and E are synthesized as described in U.S. Patent 5,003,072.

In treatment of the recipient patients in accordance with the method of the invention, the active agent can be administered by conventional drug administration procedures, preferably in formulations with pharmaceutically acceptable diluents and carriers. The active agent can be used in the free amine form or in the salt form. Pharmaceutically acceptable salt forms are illustrated, for example, by the HCl salt.

The amount of active agent to be administered must be an effective amount, that is, an amount which will be medically beneficial but does not present toxic effects which outweigh the advantages which accompany its use. It would be expected that the

average adult human daily dosage would normally range from about 0.1 mg to about 1000 mg of the active agent.

The preferable route of administration is orally in the form of capsules, tablets, syrups, elixirs and the like, although parenteral administration can also be had. Suitable formulations of the active compound in pharmaceutically acceptable diluents and carriers in therapeutic dosage form can be prepared by conventional procedures such as by reference to general texts in the field, e.g., Remington's Pharmaceutical Sciences, ed. Arthur Osol, 16th ed., 1980, Mack Publishing Co., Easton, PA, and the 18th ed., 1990. Conventional diluents and carriers are, e.g., water, normal saline, sugars, starch and the like substances.

Various other examples will be apparent to the person skilled in the art after reading the disclosure herein. All such other examples are meant to be included within the scope of the appended claims.

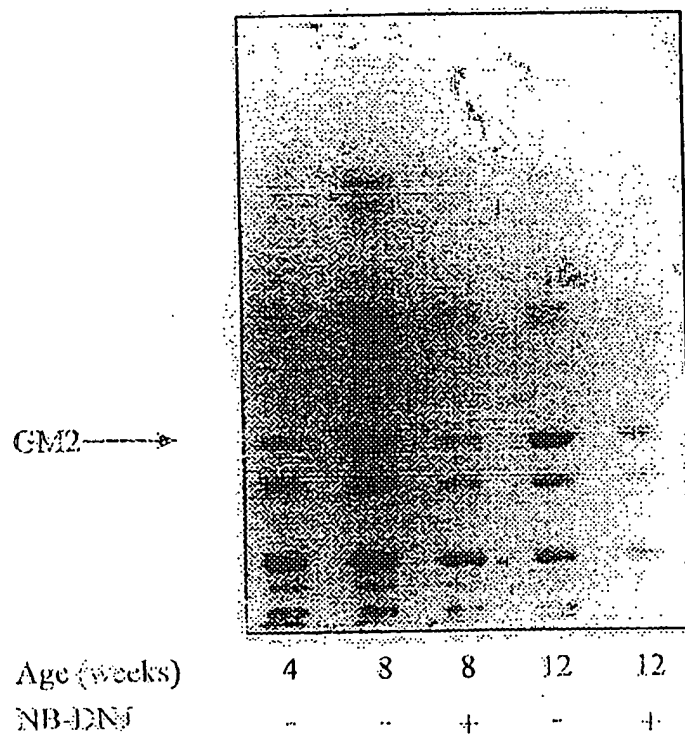
REFERENCES

1. Neufeld, E.F. (1991) *Ann. Rev. Biochem.*, 60, 257-280.
2. Beutler, E. (1992) *Science*, 256, 794-799.
3. Barton, N.W., Brady, R.O., Dambrosia, J.M., Di Bisceglie, A.M., Doppelt, S.H., Hill, S.C., Mankin, H.J., Murray, G.J., Parker, R.I., Argoff, C.E., Grewal, R.P., Yu, K-T., and Collaborators (1991) *N. Eng. J. Med.* 324, 1464-1470.
4. Beutler, E., Kay, A., Saven, A., Garver, P., Thurston, D., Dawson, A. and Rosenbloom, B. (1991) *Blood*, 78, 1183-1189.
5. Platt, F.M., Neises, G. R., Dwek, R.A. and Butters, T.D. (1994) *J. Biol Chem.*, 269, 8362-8365.
6. Platt, F.M., Neises, G. R., Dwek, R.A. and Butters, T.D. (1994) *J. Biol Chem.*, 269, 27108-27114.
7. Platt, F.M. and Butters, T.D. (1995) Inhibitors of Glycosphingolipid Biosynthesis, *Trends in Glycoscience and Glycotechnology*. 269, 495-511.
8. Yamanaka, S., Johnson, M.D., Grinberg, A., Westphal, H., Crawley, J.N., Taniike, M., Suzuki, K. and Proia, R.L. (1994) *Proc. Natl. Acad. Sci. U.S.A.*, 91, 9975-9979.
9. Sandhoff, K., Conzelmann, E., Neufeld, E.F., Kaback, M.M., and Suzuki, K. (1989) in *The Metabolic Basis of Inherited Disease*, eds. Scriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D. (McGraw-Hill, New York, Vol. 2, pp1807-1839.
10. Sango, K., Yamanaka, S. Hoffmann, A., Okuda, Y., Grinberg, A., Westphal, H., McDonald, M.P., Crawley, J.N., Sandhoff, K., Suzuki, K. and Proia, R.L. (1995) *Nature Genet.* 11, 170-176.
11. Taniike, M., Yamanaka, S., Proia, R.L., Langaman, C., Bone-Turrentine, T. and Suzuki, K. (1995) *Acta Neuropathol.* 89, 296-304.
12. Fischl, M.A., Resnick, L., Coombs, R., Kremer, A.B., Pottage, J.C., Pass, R.J., Pife, K.H., Powderly, W.G., Collier, A.C., Aspinall, R.L., Smith, S.L., Kowalski, K.G., and Wallemark, C-B. (1994) *J. AIDS* 7, 139-147.

WHAT IS CLAIMED:

1. A method for the treatment of patients having a lysosomal storage disease with a significant central nervous system (CNS) involvement comprising administering to said patient a small but storage-inhibitory effective amount of an N-alkyl derivative of a 1,5-iminosugar in which said alkyl group contains from about 2 to about 8 carbon atoms and said 1,5-iminosugar is a 1,5-dideoxy-1,5-imino-D-glucitol, or 1,5-dideoxy-1,5-imino-D-galactitol or an O-acylated prodrug of said 1,5-iminosugar.
2. The method of Claim 1 in which the 1,5-iminosugar is 1,5-dideoxy-1,5-imino-D-glucitol.
3. The method of Claim 1 in which the alkyl group is butyl.
4. The method of Claim 1 in which the 1,5-iminosugar is 1,5-dideoxy-1,5-imino-D-glucitol and the alkyl group is butyl.
5. The method of Claim 4 in which the 1,5-iminosugar is O-acylated to form the tetrabutyrates.
6. The method of any of Claims 1-5 in which the lysosomal storage disease is Tay-Sachs disease.

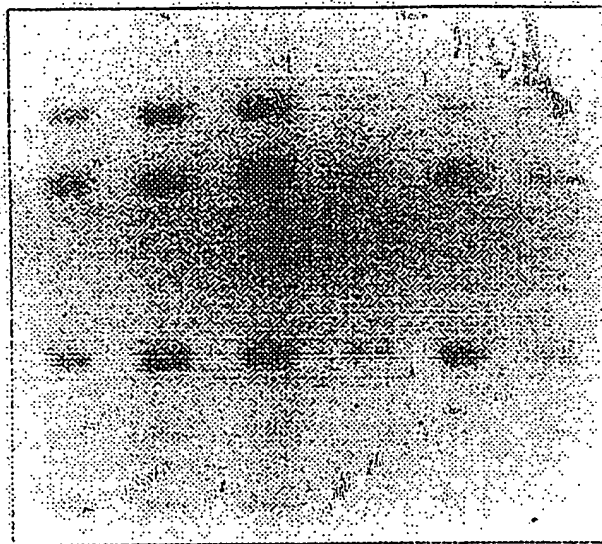
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*FIG. 1*

2/6

NB-DNJ

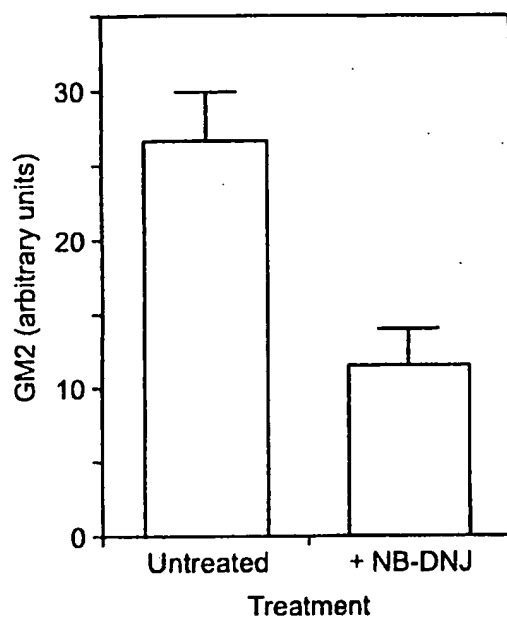
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← GM2

FIG. 2A

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*FIG. 2B*

4/6

FIG. 3A



FIG. 3B

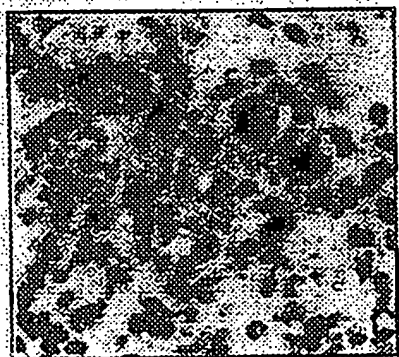
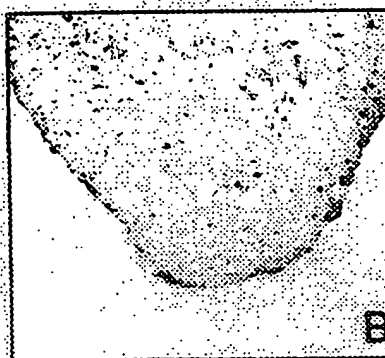


FIG. 3C

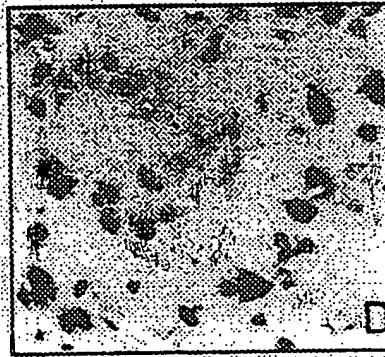


FIG. 3D

FIG. 4A



FIG. 4B



FIG. 4C

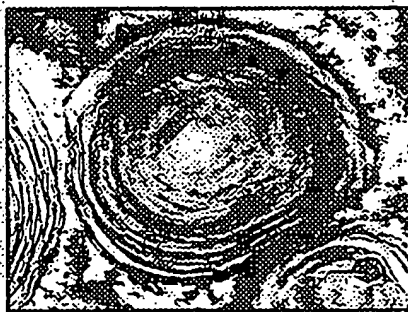


FIG. 4D

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Glycolipid Catabolism

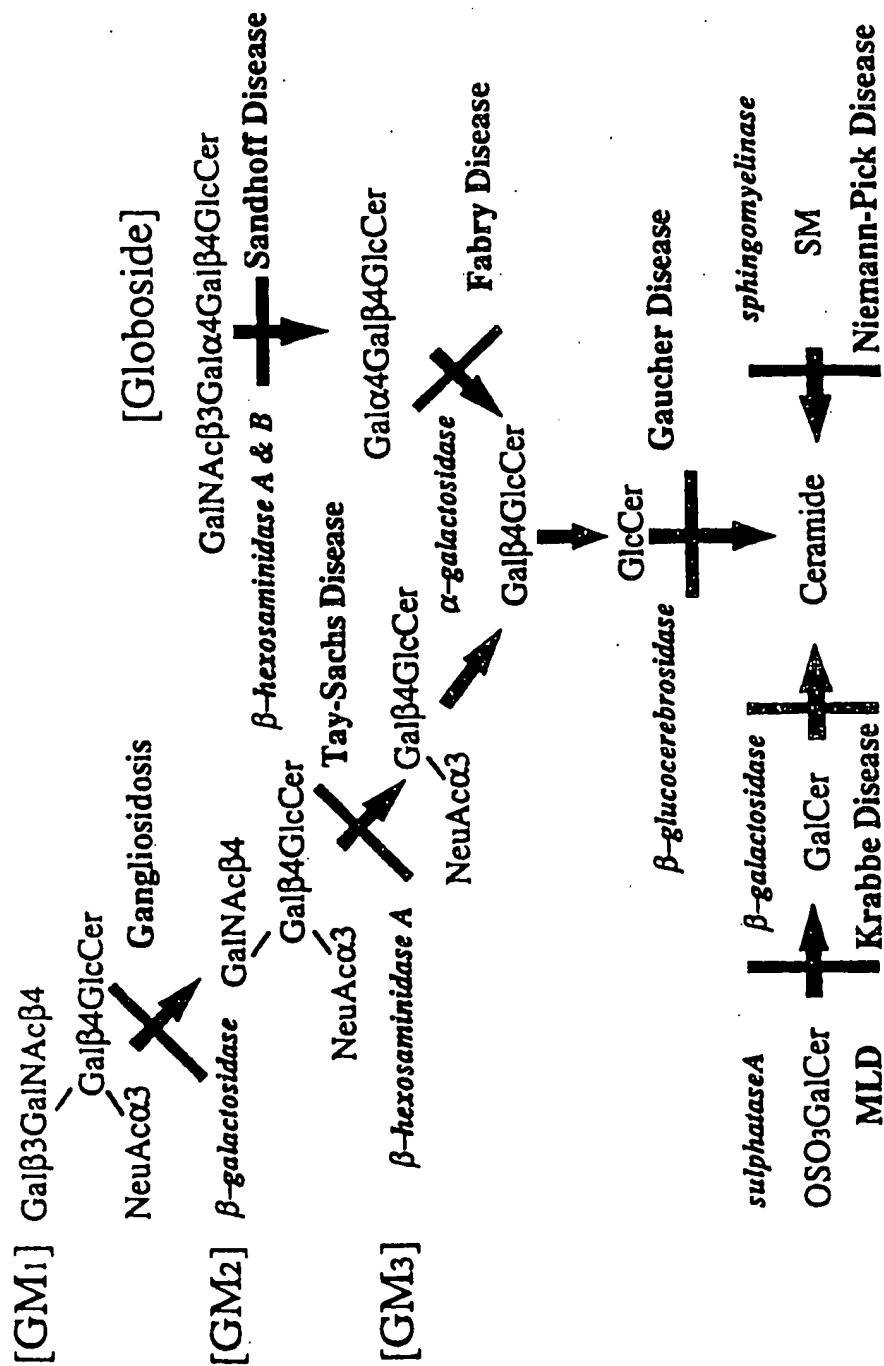


FIG. 5

INTERNATIONAL SEARCH REPORT

national Application No

PCT/US 98/00031

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/445 //A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PLATT ET AL.: "Prevention of lysosomal storage in Tay-Sachs mice treated with N-butyldeoxynojirimycin" SCIENCE, vol. 276, no. 5311, 18 April 1997, pages 428-431, XP002065772 see the whole document --- -/--	1-4,6

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

25 May 1998

Date of mailing of the international search report

11.06.98

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PLATT ET AL.: "Extensive glycosphingolipid depletion in the liver and lymphoid organs of mice treated with N-butyldeoxynojirimycin" J. BIOL. CHEM., vol. 272, no. 31, 1 August 1997, pages 19365-19372, XP002065773 see page 19365, right-hand column, paragraph 3 see page 18371, left-hand column, paragraph 2 see page 19372, left-hand column, paragraph 2	1-6
P,X	--- KOLTER ET AL.: "A chemical concept for the treatment of Tay-Sachs disease" ANGEW. CHE., INT. ED. ENGLAND, vol. 36, no. 18, 2 October 1997, pages 1955-1959, XP002065774 see the whole document	1-4,6
X	--- WO 94 26714 A (G.D. SEARLE & CO.) 24 November 1994 cited in the application see the whole document, especially pages 2-4, 16-21, and page 25 lines 16-21	1-5
Y		6
X	--- DANIEL ET AL.: "Evidence for processing of dolichol-linked oligosaccharides in patients with neuronal ceroid-lipofuscinosis" AM. J. MED. GENET., vol. 42, no. 4, 15 February 1992, pages 586-592, XP002065775 see page 586 see page 590, right-hand column, last paragraph	1,2
Y	--- LIESSEM ET AL.: "Synthese von 2-acetamido-1,4-imino-1,2,4-tridesoxy-D-Ga laktitol und kompetitive Inhibition der humanen lysosomalen beta-Hexosaminidase A" CARBOHYDRATE RES., vol. 250, no. 1, 1993, pages 19-30, XP002065776 see page 20	6
A	--- -/--	1-5

INTERNATIONAL SEARCH REPORT

national Application No

PCT/US 98/00031

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PLATT ET AL.: "N-butyldeoxygalactonojirimycin inhibits glycolipid biosynthesis but does not affect N-linked oligosaccharide processing" J. BIOL. CHEM., vol. 268, no. 43, 28 October 1994, pages 27108-27115, XP002065777 see the whole document</p>	1-6
A	<p>PLATT F M ET AL: "N-BUTYLDEOXYNOJIRIMYCIN IS A NOVEL INHIBITOR OF GLYCOLIPID BIOSYNTHESIS" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 11, 18 March 1994, pages 8362-8365, XP000615445</p>	1-4,6
A	<p>OSIECKI-NEWMAN K ET AL: "HUMAN ACID -GLUCOSIDASE: USE OF INHIBITORS ALTERNATIVE SUBSTRATES AND AMPHIPHILES TO INVESTIGATE THE PROPERTIES OF THE NORMAL AND GAUCHER DISEASE ACTIVE SITES" BIOCHIMICA ET BIOPHYSICA ACTA, vol. 915, no. 1, 1 January 1987, pages 87-100, XP000561731</p>	1-4
A	<p>LEMBCKE ET AL.: "Lysosomal storage of glycogen as a sequel of alpha-glucosidase inhibition by the absorbed deoxynojirimycin derivative emiglitate (BAYol248)" RES. EXP. MED. , vol. 191, no. 6, 1991, BERLIN, pages 389-404, XP002065778 see the whole document</p>	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/00031

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-6 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

national Application No

PCT/US 98/00031

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9426714 A	24-11-1994	US 5399567 A	21-03-1995
		AT 148456 T	15-02-1997
		AU 6783294 A	12-12-1994
		CA 2159988 A	24-11-1994
		DE 69401658 D	13-03-1997
		DE 69401658 T	12-06-1997
		EP 0698012 A	28-02-1996
		ES 2097653 T	01-04-1997
		JP 8510244 T	29-10-1996
		US 5472969 A	05-12-1995
		US 5656641 A	12-08-1997
		US 5580884 A	03-12-1996
		US 5525616 A	11-06-1996

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<p>(21) International Application Number: PCT/US98/23239 (22) International Filing Date: 9 November 1998 (09.11.98) (30) Priority Data: 60/065,051 10 November 1997 (10.11.97) US (71) Applicant (for all designated States except US): G.D. SEARLE & CO. [US/US]; Corporate Patent Dept., P.O. Box 5110, Chicago, IL 60680-5110 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): JACOB, Gary, S. [US/US]; 12541 Mason Forest Drive, Creve Coeur, MO 63141 (US). (74) Agents: WILLIAMS, Roger, A. et al.; G.D. Searle & Co., Corporate Patent Dept., P.O. Box 5110, Chicago, IL 60680-5110 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: USE OF ALKYLATED IMINOSUGARS TO TREAT MULTIDRUG RESISTANCE</p> <p>(57) Abstract</p> <p>The present invention relates to the field of cancer chemotherapy. More particularly, the present invention relates to a compound for improving the effectiveness of cancer chemotherapy by preventing, reducing, or reversing the development of cellular resistance to chemotherapeutic agents, i.e., the phenomenon known as "multidrug resistance" (MDR), during the course of therapy. This is achieved by administering to patients N-alkyl-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds ("iminosugars") in conjunction with chemotherapeutic drugs.</p>		

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USE OF ALKYLATED IMINOSUGARS TO TREAT MULTIDRUG RESISTANCE

BACKGROUND OF THE INVENTIONField of the Invention

The present invention relates to the field of cancer chemotherapy. More particularly, the present invention relates to a compound for improving the effectiveness of cancer chemotherapy by preventing, reducing, or reversing the development of cellular resistance to chemotherapeutic agents, i.e., the phenomenon known as "multidrug resistance" (MDR), during the course of therapy. This is achieved by administering to patients N-alkyl-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds ("iminosugars") in conjunction with chemotherapeutic drugs.

Description of Related ArtMultidrug Resistance (MDR)

Multidrug resistance, the phenomenon whereby primary exposure of tumor cells to a single chemotherapeutic drug results in cellular resistance to

multiple drugs, is believed to be the basis for tumor cell survival (Bradley et al. (1988) *Biochim. Biophys. Acta* 948:87-128). MDR is manifested as a simultaneously acquired cellular resistance to several cytotoxic substances, which can be surprisingly structurally and functionally unrelated, and is often observed after prolonged exposure of cells to anticancer drugs of the "multidrug resistance group." The latter includes such different compounds as actinomycin D, mitomycin C, anthracyclines, colchicine, rhodamine, ethidium bromide, doxorubicin, epipodophyllotoxins, paclitaxel, taxol, reserpine, and the vinca alkaloids. Exposure of cells to one of these drugs can lead not only to specific resistance to this drug, but also to non-specific cross-resistance to all the other drugs of the MDR group.

Study of this phenomenon has focused on a number of different possible biological mechanisms. Volm et al. ((1993) *Cancer* 71:2981-2987) and Bradley et al. ((1994) *Cancer Metastasis Rev.* 13:223-233) have investigated the overexpression of P-gp, a plasma membrane glycoprotein believed to rapidly efflux MDR-type drugs, thus protecting cells from damage by preventing these drugs from reaching their intracellular targets. Doige et al. ((1993) *Biochim. Biophys. Acta* 1146:65-72) and Wadkins et al. ((1993) *Biochim. Biophys. Acta* 1153:225-236) have studied the role of lipids in MDR. While differences in the glycerolipid and sphingomyelin compositions of MDR and drug-sensitive

cells have been observed (Holleran et al. (1986) *Cancer Chemother. Pharmacol.* 17:11-15; Ramu et al. (1984) *Cancer Treat. Rep.* 68:637-641; May et al. (1988) *Int. J. Cancer* 42:728-733; Welsh et al. (1994) *Arch. Biochem. Biophys.* 315:41-47; Wright et al. (1985) *Biochem. Biophys. Res. Commun.* 133:539-545), and the ganglioside composition of MDR and drug-sensitive cells has been investigated, no clear picture as to the basis of drug resistance emerged from these studies.

More recently, Lavie et al. ((1996) *J. Biol. Chem.* 271:19530-10536) demonstrated a correlation between the cellular content of glycosphingolipids and MDR. These workers demonstrated that tamoxifen, verapamil, and cyclosporin A, agents that reverse multidrug resistance, as well as 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol, an inhibitor of glucosylceramide synthesis, decrease glucosylceramide levels in an MDR human breast cancer cell line that accumulates high levels of glucosylceramide compared with the parental wild-type, drug-sensitive cell line (Lavie et al. (1997) *J. Biol. Chem.* 272:1682-1687). They concluded that high cellular levels of glucosylceramide are correlated with MDR, and that glycolipids are therefore a target for the action of MDR-reversing agents.

1,5-dideoxy-1,5-imino-D-glucitol and galactitol
Compounds

1,5-dideoxy-1,5-imino-D-glucitol (also known as 1-deoxynojirimycin, DNJ) and its *N*-alkyl derivatives are known inhibitors of the *N*-linked oligosaccharide processing enzymes α -glucosidase I and II (Saunier et al., *J. Biol. Chem.* (1982) 257:14155-14161 (1982); Elbein, *Ann. Rev. Biochem.* (1987) 56:497-534). As glucose analogs, they were also predicted to have the potential to inhibit glucose transport, glucosyltransferases, and/or glycolipid synthesis (Newbrun et al., *Arch. Oral Biol.* (1983) 28: 516-536; Wang et al., *Tetrahedron Lett.* (1993) 34:403-406). Their inhibitory activity against glucosidases has led to the development of these compounds as anti-hyperglycemic agents and antiviral agents. See, for example, PCT International Publication WO 87/03903 and U.S. Patents 4,065,562; 4,182,767; 4,533,668; 4,639,436; 4,849,430; 4,957,926; 5,011,829; and 5,030,638. *N*-butyl DNJ is an inhibitor of HIV replication *in vitro* (Fleet et al. (1988) *FEBS Lett.* 237:128-132; Karpas et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:9229-9233). This compound has been clinically evaluated as a potential AIDS therapeutic (Jacob et al. (1992) in *Natural Products as Antiviral Agents*, C. K. Chu et al., Eds., pp. 137-152, Plenum Publishing Co., N.Y.), and has been found to exhibit little cytotoxicity *in vitro* (Platt et al. (1992) *Eur. J. Biochem.* 208:187-193).

Platt et al. ((1994) *J. Biol. Chem.* 269:8362-8365) have demonstrated that certain *N*-alkylated derivatives of DNJ inhibit the glucosyltransferase-catalyzed biosynthesis of glucosylceramide, resulting in the inhibition of biosynthesis of all glucosylceramide-based glycosphingolipids. Glycolipids constitute an important class of glycoconjugates found in the membranes, and particularly the plasma membrane, of eukaryotic cells. These authors speculated that these

N-alkylated derivatives specifically inhibit UDP-glucose-*N*-acylsphingosine glucosyltransferase (EC 2.4.1.80). This transferase generates glucosylceramide (GlcCer), the precursor for the more complex glycosphingolipids and gangliosides. Platt et al. also demonstrated that *N*-butyl DNJ inhibited glycolipid expression at the cell surface. The authors suggested that *N*-alkylated DNJs would be useful in treating lysosomal glycolipid storage disorders such as Gaucher's disease.

In a subsequent paper, Platt et al. showed that the galactose analogue of *N*-butyl DNJ, i.e., *N*-butyl-deoxygalactonojirimycin (*N*-butyl DGJ), is a more selective inhibitor of glycolipid biosynthesis, only weakly inhibiting the *N*-linked oligosaccharide processing enzymes α -glucosidases I and II, and not inhibiting lysosomal β -glucocerebrosidase (which is required for the cleavage of GlcCer to glucose and ceramide). *N*-butyl DGJ was shown to be comparable to *N*-butyl DNJ as an inhibitor of UDP-

glucose-*N*-acylsphingosine glucosyltransferase and in preventing lysosomal glycolipid storage in an *in vitro* model of Gaucher's disease.

5 In 1997, Platt et al. (Science 276:428-431) reported the prevention of glycosphingolipid lysosomal storage in a mouse model of Tay-Sachs disease using *N*-butyl DNJ. This disease is characterized by a deficiency in the A isoenzyme of β -hexosaminidase, which degrades G_{M2} ganglioside. A deficiency of this enzyme in humans results
10 in accumulation of G_{M2} ganglioside in brain cell lysosomes, leading to severe neurological degeneration. The authors noted that this compound is water soluble and noncytotoxic over a broad range of concentrations *in vitro* and *in vivo*. Oral administration to healthy mice resulted in
15 glycosphingolipid depletion in multiple organs without causing any overt pathological side effects. In Tay-Sachs mice, no toxicity to *N*-butyl DNJ was observed based on visible inspection and observation of the animals, and of organ weights at autopsy. While spleen and thymus tissues
20 were 50% acellular, no immunocompromization was apparent. The authors concluded that in this *in vivo* mammalian model, oral treatment with *N*-butyl DNJ is well tolerated, and effectively inhibits glycosphingolipid biosynthesis and subsequent accumulation in brain cell lysosomes.

Treatment of MDR

Many chemosensitizers have been reported to antagonize MDR in in vitro systems, and some have been shown to be effective in vivo when coadministered with appropriate chemotherapeutic agents to nude mice bearing multidrug-resistant tumors. Unfortunately, success in the laboratory has not necessarily translated to success in the clinic. Dose-limiting side effects of first-generation MDR modulators have been observed. Low therapeutic indices and failure to achieve therapeutic blood levels have also been problematic (Dalton et al. (1995) *Cancer* 75:815-20; Tsuro et al. (1981) *Cancer Res.* 41:1967-72; Ries et al. (1991) *Med. Oncol. Tumor Pharmacother.* 9:39-42; Chabner (1991) *J. Clin. Oncol.* 9:4-6; Raderer et al. (1993) *Cancer* 72:3553-63; Mulder et al. (1996) *J. Exp. Ther. Oncol.* 1:19-28; Fischer et al. (1995) *Hematol. Oncol. Clin. North Am.* 9:363-82; Wishart et al. (1994) *J. Clin. Oncol.* 9:1771-77). In addition, patient dosing is sometimes complicated by pharmacokinetic drug interactions, resulting in increased plasma concentrations or decreased elimination of cytotoxic drugs, resulting in increased toxicity (Egorin et al. (1996) *Proc. Am. Soc. Clin. Oncol.* 15:473; Beketic-Oreskovic et al. (1995) *J. Natl. Cancer Inst.* 1593-602.88). Most of the results from MDR-reversal trials have been disappointing, except for those for some hematological cancers (Chabner (1991) *J. Clin. Oncol.* 9:4-6; Raderer et al. (1993) *Cancer* 72:3553-63; Mulder et al. (1996) *J. Exp.*

Ther. Oncol. 1:19-28; Fischer et al. (1995) *Hematol. Oncol. Clin. North Am.* 9:363-82).

Thus, a common, major obstacle to cure with chemotherapeutic agents is the survival and continued proliferation of cells that are resistant to further treatment. MDR is therefore a formidable impediment to successful chemotherapy. The art continues to seek agents that can be used to prevent or reduce this phenomenon during cancer chemotherapy. The use of *N*-substituted-imino-D-glucitol or galactitol derivatives in conjunction with chemotherapeutic agents for preventing or reducing the extent of MDR during chemotherapy has not, as far as the present inventor is aware, been previously disclosed or suggested.

SUMMARY OF THE INVENTION

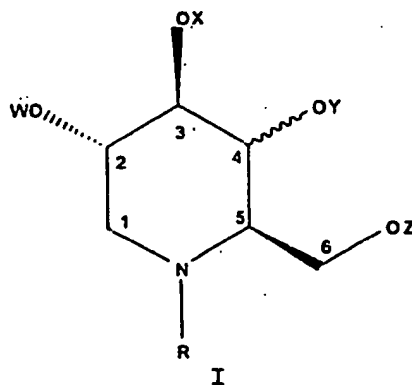
In response to the need of the healing arts for agents that can be used to avoid the deleterious consequences of MDR during chemotherapy, the present inventor has surprisingly discovered that certain iminosugar glucosylceramide synthase inhibitors are effective for this purpose. These inhibitors can be used to prevent, reduce, or reverse MDR often observed during treatment of cancer patients with chemical anti-cancer agents.

As noted above, first-generation MDR modulators exhibit a number of disadvantageous side effects. In

addition, drugs such as verapamil, tamoxifen, cyclosporin A, and 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol exhibit other, well known pharmacologic effects which may be undersirable in certain patients. In contrast, the iminosugars of the present invention possess beneficial advantages in treating MDR including, but not limited to, mechanistic specificity, lack of drug-drug interactions, and minimal or no effect on elimination of cytotoxic chemotherapeutic drugs.

Accordingly, in one aspect, the present invention provides a compound for preventing, reducing, or reversing multidrug resistance in a patient undergoing treatment with a chemotherapeutic agent, comprising

an anti-multidrug resistance effective amount of an N-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compound, or pharmaceutically acceptable salt thereof, of Formula I:



wherein R is selected from arylalkyl, cycloalkylalkyl, and branched or straight chain alkyl having a chain length of C₂ to C₂₀, and W, X, Y and Z are each independently selected

from hydrogen, alkanoyl, aroyl, and trifluoroalkanoyl,

Preferred compounds are those wherein R is n-butyl or n-hexyl.

The N-substituted-1,5-dideoxy-
5 1,5-imino-D-glucitol or galactitol compound, or combinations thereof, can be administered in accordance with a variety of different regimens, including prior to administration of the chemotherapeutic agent; both prior to
10 and simultaneously with administration of the chemotherapeutic agent; prior to, simultaneously with, and subsequently to administration of the chemotherapeutic agent; simultaneously with administration of the chemotherapeutic agent; prior to and subsequently to
15 administration of the chemotherapeutic agent; or daily throughout the entire course of treatment with the chemotherapeutic agent.

In the preferred method about 1,000 mg/day to about 3,000 mg/day of N-(n-butyl)-1,5-dideoxy-1,5-imino-D-glucitol or
20 galactitol or N-(n-hexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol, or a pharmaceutically acceptable salt thereof, daily throughout the course of administration of a chemotherapeutic agent selected from an anthracycline, an alkaloid, an anti-microtubule drug, a topoisomerase II
25 inhibitor, and a DNA damaging agent. Administration of the

N-alkylated iminosugar can commence about 14 days prior to administration of the chemotherapeutic agent.

In another aspect, the present invention provides a pharmaceutical composition, comprising an anti-multidrug resistance effective amount of at least one *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compound as above, an anti-tumor effective amount of at least one anti-tumor chemotherapeutic compound, and a pharmaceutically acceptable carrier.

Further scope of the applicability of the present invention will become apparent from the detailed description and drawings provided below. However, it should be understood that the following detailed description and examples, while indicating preferred embodiments of the invention, are given by way of illustration only since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DETAILED DESCRIPTION OF THE INVENTION

The following detailed description is provided to aid those skilled in the art in practicing the present invention. Even so, this detailed description should not be construed to unduly limit the present invention as modifications and variations in the embodiments discussed herein can be made by those of ordinary skill in the art

without departing from the spirit or scope of the present inventive discovery.

The contents of each of the references cited herein, including the contents of the references cited within these primary references, are herein incorporated by reference in their entirety.

The present inventor has discovered that *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol and galactitol compounds used in conjunction with antineoplastic chemotherapeutic agents are effective in preventing the development of, reducing the extent of, or reversing MDR in patients receiving chemotherapy.

The present invention thus provides pharmaceutical compositions for preventing or reducing MDR in humans and other mammals being treated with chemical antitumor compounds by administering one or more *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds to patients. The iminosugar and chemotherapeutic drugs of this invention can be provided to cells, tissues, or organs *in vitro* or *in vivo*, or to a human or other mammalian patient, including domestic animals such as cats and dogs, either in separate pharmaceutically acceptable formulations, formulations containing more than one therapeutic agent, or by an assortment of single agent and multiple agent formulations. However administered, these drug combinations form an anti-MDR effective and chemotherapeutically effective amount of

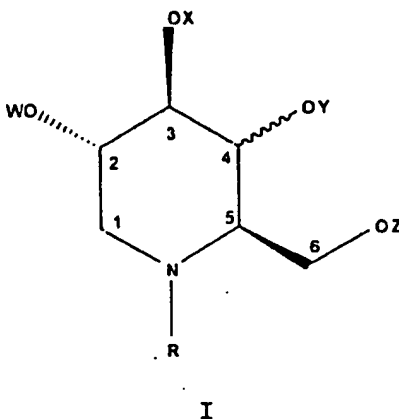
components. Administration of the present iminosugar and chemotherapeutic drugs to cells, tissues, or organs in vitro can be used as model experimental systems in which to investigate the phenomenon of MDR, with the goal of optimizing in vivo treatment therefor.

As used herein, the term "anti-MDR effective amount" refers to an amount of an *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compound, or combination thereof, effective in preventing the development of, reducing the extent of, or reversing multidrug resistance often observed in tumor cells of patients being treated with antineoplastic agents. Such effective amount is medically beneficial, and does not cause toxic effects that outweigh the advantages associated with the use of these *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds in overcoming the adverse effects of MDR. The ultimate result is enhanced effectiveness of the chemotherapy.

Also as used herein, the term "multidrug resistance group" refers to those antineoplastic agents to which tumor cells develop resistance after exposure thereof to an anticancer chemotherapeutic compound, i.e., to which such tumor cells develop multidrug resistance, whether this be specific resistance to this particular anticancer chemotherapeutic compound, or non-specific cross-resistance to other chemotherapeutic compounds which may or may not be structurally and functionally related.

N-substituted-1,5-dideoxy-1,5-imino-D-glucose and Galactose
Compounds

N-substituted-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol compounds useful in the present invention are
represented by formula I:



The glucitol and galactitol stereoisomers
encompassed by formula I differ in the orientation of the
hydroxyl group on C-4 of the ring. Employing the
convention of Fleet et al. ((1992) *Glycobiology* 2:199-210),
the ring in formula I lies flat in the plane of the page.
A group attached to the ring via a bond depicted with a
series of dashed lines is oriented below the plane of the
ring; a group attached to the ring via a bond depicted with
a solid, elongated triangle is oriented above the plane of
the ring. The group attached to the ring at C-4 via the
bond depicted by the squiggly line is either below the
plane of the ring (glucitol derivatives) or above the plane
of the ring (galactitol derivatives).

In formula I, R is selected from arylalkyl, cycloalkylalkyl, and branched or straight chain alkyl having a chain length of C₂ to C₂₀, preferably C₄ to C₂₀, more preferably C₄ to C₁₄, more preferably C₄ to C₁₀, more preferably C₄ to C₈, and most preferably C₄ to C₆ in the principal chain. n-butyl and n-hexyl are preferred.

R can also be C₁ to C₂₀ alkyl, preferably C₂ to C₁₄, more preferably C₆ to C₁₂, more preferably C₄ to C₁₀ alkyl, containing 1 to 5, more preferably 1 to 3, most preferably 1 to 2, oxygen atoms, i.e., oxa derivatives. Preferred R oxa derivatives are 3-oxanonyl, 3-oxadecyl, 7-oxanonyl, and 7-oxadecyl.

W, X, Y and Z are independently selected from hydrogen, alkanoyl, aroyl, and trifluoroalkanoyl.

As used herein, the term "alkyl" as used in "arylalkyl" and "cycloalkylalkyl," either unsubstituted or containing the various substituents defined herein, can contain from one to about six carbon atoms in the principal chain, and up to about 15 carbon atoms total. Such alkyl groups include, for example, methyl, ethyl, propyl, isopropyl, butyl, hexyl, cyclopropyl, cyclopentyl, cyclohexyl, and the like. Substituents of the substituted alkyl groups described herein can include, for example, groups selected from alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, O, S, N, P, or halogen (Cl, F, Br, or I) atoms. Optionally, these substituent alkyl, cycloalkyl, etc., groups can be substituted with O, S, N, P, or halogen

(Cl, F, Br, or I) atoms. These substituent alkyl, cycloalkyl, etc., groups include, for example, lower alkoxy groups such as methoxy, ethoxy, and butoxy, and groups such as halo, nitro, amino, and keto.

5 The alkenyl groups described herein, either unsubstituted or with the various substituents defined herein, are preferably lower alkenyl groups containing from about two to about six carbon atoms in the principal chain, and up to about 15 carbon atoms total. They can be
10 substituted, straight, or branched chain, and include ethenyl, propenyl, isopropenyl, butenyl, isobutenyl, hexenyl, and the like.

 The alkynyl groups described herein, either unsubstituted or with the various substituents defined
15 herein, are preferably lower alkynyl groups containing from about two to about six carbon atoms in the principal chain, and up to about 15 carbon atoms total. They can be substituted, straight or branched chain, and include ethynyl, propynyl, butynyl, isobutynyl, hexynyl, and the
20 like.

 The aryl moieties described herein, either unsubstituted or with various substituents defined herein, can contain from about 6 to about 15 carbon atoms, and include phenyl and naphthyl. Substituents include
25 alkanoxy, protected hydroxy, halogen, alkyl, aryl, alkenyl, acyl, acyloxy, nitro, amino, amido, etc. Phenyl is a preferred aryl.

The cycloalkyl moieties described herein, either unsubstituted or with various substituents defined herein, can contain from about 5 to about 15 atoms, and include cyclobutylbutyl, cyclohexylhexyl, and the like. Substituents include alkanoxy, protected hydroxy, halogen, alkyl, aryl, alkenyl, acyl, acyloxy, nitro, amino, and amido.

The alkanoyl groups, either unsubstituted or substituted with the various substituents defined hereinabove for "alkyl" groups, and the trifluoroalkanoyl groups described herein, can contain from one to about six carbon atoms in the principal chain, and up to about 15 carbon atoms total, and include acetyl, propanoyl, butanoyl, and the like. The aroyl groups described herein, either unsubstituted or with various substituents defined herein, can contain from about 6 to about 15 carbon atoms, and include benzoyl. Substituents include alkanoxy, protected hydroxy, halogen, alkyl, aryl, alkenyl, acyl, acyloxy, nitro, amino, amido, etc. Benzoyl is a preferred aroyl.

The carbon atoms, i.e., the methyl and methylene groups, constituting the principal backbone of the branched or straight chain alkyl groups having a chain length of C₁ to C₂₀, can also be substituted as variously described above.

Representative *N*-substituted-imino-D-glucitol and galactitol compounds useful in the present invention include, but are not limited to:

- N*-(*n*-ethyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- N*-(*n*-propyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- 5 *N*-(*n*-butyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- N*-(*n*-hexyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- 10 *N*-(*n*-heptyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- N*-(*n*-octyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- N*-(*n*-octyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrates;
- 15 *N*-(*n*-nonyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrates;
- N*-(*n*-decyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrates;
- N*-(*n*-undecyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrates;
- 20 *N*-(*n*-nonyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- N*-(*n*-decyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- 25 *N*-(*n*-undecyl-)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;

- N*-(*n*-dodecyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- N*-(2-ethylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- 5 *N*-(4-ethylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- N*-(5-methylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- N*-(3-propylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- 10 *N*-(1-pentylpentylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- N*-(1-butylbutyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- 15 *N*-(7-methyloctyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- N*-(8-methylnonyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- N*-(9-methyldecyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- 20 *N*-(10-methylundecyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- N*-(6-cyclohexylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- 25 *N*-(4-cyclohexylbutyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;

- N*-(2-cyclohexylethyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- N*-(1-cyclohexylmethyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- 5 *N*-(1-phenylmethyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- N*-(3-phenylpropyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- 10 *N*-(3-(4-methyl)-phenylpropyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- N*-(6-phenylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;
- N*-(*n*-nonyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol, tetrabutyrates;
- 15 *N*-(*n*-decyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol, tetrabutyrates;
- N*-(*n*-undecyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol, tetrabutyrates;
- 20 *N*-(*n*-dodecyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol, tetrabutyrates;
- N*-(2-ethylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol, tetrabutyrates;
- N*-(4-ethylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol, tetrabutyrates;
- 25 *N*-(5-methylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol, tetrabutyrates;

- N*-(3-propylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N*-(1-pentylpentylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- 5 *N*-(1-butylbutyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N*-(7-methyloctyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- 10 *N*-(8-methylnonyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N*-(9-methyldecyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N*-(10-methylundecyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- 15 *N*-(6-cyclohexylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N*-(4-cyclohexylbutyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N*-(2-cyclohexylethyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- 20 *N*-(1-cyclohexylmethyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N*-(1-phenylmethyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- 25 *N*-(3-phenylpropyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;

N-(3-(4-methyl)-phenylpropyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol, tetrabutyrates; and
N-(6-phenylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol, tetrabutyrates.

5 Pharmaceutically acceptable salts of any of the glucitol or galactitol compounds encompassed herein can also be used in the methods of the present invention.

 Preferred compounds are *N*-(*n*-butyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol and *N*-(*n*-hexyl)-1,5-
10 dideoxy-1,5-imino-D-glucitol or galactitol.

 The *N*-substituted-imino-D glucitol compounds useful in the present invention can be prepared by methods well known in the art as described in, for example, Fleet et al. (1988) *FEBS Lett.* 237:128-132, U.S. Patents Nos.
15 4,182,767, 4,639,436, and 5,003,072, as well as PCT International Publication WO 95/19172 and the references cited therein. Deoxynojirimycin (DNJ) can be obtained from Sigma Chemical Company (St. Louis; cat. no. D 3291).

N-substituted-imino-D-galactitol compounds can be
20 prepared from deoxygalactonojirimycin (DGJ), which can be obtained from Cambridge Research Biochemicals (Northwich, Cheshire, U.K.), as described in Platt et al. (1994) *J. Biol. Chem.* 269:27108-27114. Briefly, DGJ can be reductively *N*-alkylated in the presence of palladium black
25 under hydrogen using the appropriate aldehyde by the method of Fleet et al. (1988) *FEBS Lett.* 237:128-132. The reaction mixture is filtered through Celite, and the

solvent removed by evaporation under vacuum. The resulting *N*-alkylated analogues are then purified by ion-exchange chromatography (Dowex® AG50-X12, H⁺ form) in 2M aqueous ammonia, and the solvent removed by evaporation. The compounds can then be lyophilized and analyzed by 1D ¹H NMR and by matrix-assisted laser desorption.

Methods for introducing oxygen into alkyl side chains are disclosed in Tan et al., (1994) *Glycobiology* 4(2):141-149.

Non-limiting illustrative preparative procedures are presented below in Examples 1-5.

In treating MDR, the medical practitioner can use the *N*-substituted-imino-D-glucitol or galactitol compounds of this invention in the form of pharmaceutically acceptable salts. Such salts must clearly have a pharmaceutically acceptable anion or cation. Suitable pharmaceutically acceptable acid addition salts of the compounds of the present invention can be derived, when possible, from inorganic acids such as hydrochloric, hydrobromic, hydroiodic, phosphoric, metaphosphoric, nitric, sulfonic, and sulfuric acids, and organic acids such as acetic, adipic, alginic, aspartic, benzoic, benzenesulfonic, bisulfatic, butyric, camphoric, camphorsulfonic, citric, digluconic, cyclopentane-propionic, dodecylsulfatic, ethanesulfonic, gluconic, glycolic, glucoheptanoic, glycerophosphatic, hemisulfatic, heptanoic, hexanoic, fumaric, 2-hydroxy-ethanesulfonic,

lactic, maleic, malic, methanesulfonic, nicotinic, 2-naphthalenesulfonic, oxalic, palmitic, pectinic, persulfatic, 3-phenylpropionic, picric, pivalic, propionic, succinic, tartaric, thiocyanic, toluenesulfonic, tosylic, mesylic, and undecanoic. The chloride salt is particularly preferred for medical purposes.

The present *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds have basic nitrogen atoms, and can be used in the form of a pharmaceutically acceptable salt thereof. The basic nitrogen-containing groups can be quaternized with agents such as lower alkyl halides, such as methyl, ethyl, propyl, and butyl chloride, bromides, and iodides; dialkyl sulfates such as dimethyl, diethyl, dibutyl, and diamyl sulfates; long chain halides such as decyl, lauryl, myristyl, and stearyl chlorides, bromides, and iodides; aralkyl halides such as benzyl and phenethyl bromides, and others. Water- or oil-soluble or dispersible products are thereby obtained as desired. The salts are formed by combining the basic compounds with the desired acid.

Other compounds of this invention that are acids can also form salts. Examples include salts with alkali metals or alkaline earth metals, such as sodium, potassium, calcium, or magnesium, or with organic bases or basic quaternary ammonium salts.

Compounds of this invention can be acids or bases. As such, they can be used to form salts with one another. This type of salt can then be provided to the

patient in a pharmaceutically acceptable formulation or as a pure single salt.

Chemotherapeutic Agents

As indicated below, there are a large number of antineoplastic agents available in medical use, in clinical evaluation, and in pre-clinical development, that can be employed in the treatment of tumor cell growth in conjunction with the *N*-substituted-imino-D-glucitol or galactitol compounds of the present invention. Such antineoplastic agents fall into a number of major categories, including antibiotics (such as actinomycin D), antimetabolites, anthracyclines, alkaloids, alkylating agents, anti-microtubule agents (such as the vinca alkaloids and taxol), anti-tumor enzymes, hormonal agents, immunological agents, interferon-type agents, platinum-containing agents, topoisomerase inhibitors, DNA damaging agents (agents that cause breaks, such as single strand breaks, in DNA), and a category of miscellaneous agents. An example of a compound of this last category is carbetimer, which is an antineoplastic agent having significant cytotoxic activity in clonogenic assays (Kisner et al. (1983) *Proc. ASCO* 2) and in nude mice bearing a variety of human tumors (Ardalan et al. (1986) *Cancer Res.* 46).

Antineoplastic Compounds

17-Beta-Estradiol
Aclarubicin
Aldesleukin
5 Allopurinol
Altretamine
Amifostine
Amsacrine
Anastrozole
10 Asparaginase
Azidopine
BCG vaccine
BCNU
Bicalutamide
15 Bleomycin Sulfate
Busulfan
Carboplatin
Carmustine
Chlorambucil
20 Cisplatin
Cladribine
Clodronate disodium
Cyclophosphamide
Cytarabine
25 Cytarabine ocfosfate
Dacarbazine
Dactinomycin

Daunorubicin Hydrochloride
Dexrazoxane
Diethylstilbestrol
Docetaxel
5 Doxorubicin Hydrochloride
Dronabinol
Eflornithine
Erythropoietin
Estramustine Phosphate Sodium
10 Etidronate Disodium
Etoposide
Etoposide phosphate
Fadrozole
Filgrastim
15 Fluasterone
Fludarabine Phosphate
Fluorouracil
Fluoxymesterone
Flutamide
20 Fluxuridine
Formestane
Fotemustine
Gallium Nitrate
Gemcitabine
25 Gemcitabine Hydrochloride
Goserelin Acetate
Granisetron Hydrochloride
Hexadecylphosphocholine

Hydroxyurea
Idarubicin
Idarubicin Hydrochloride
Ifosfamide
5 Interferon alfa-2a
Interferon alfa-2b
Interferon, Toray (beta)
Irinotecan
Irinotecan Hydrochloride
10 Lentinan
Letrozole
Leucovorin Calcium
Leuprolide Acetate
Levamisole
15 Lomustine
Lonidamine
Mechlorethamine Hydrochloride
Medroxyprogesterone Acetate
Megestrol Acetate
20 Melphalan
Mercaptopurine
Methotrexate Sodium
Mitolactol
Mitomycin
25 Mitotane
Mitoxantrone Hydrochloride
Nedaplatin
Nilutamide

Octreotide Acetate
Ondansetron Hydrochloride
Oxaliplatin
Paclitaxel
5 Pamidronate Disodium
Pegasparagase
Pegaspargase
Pentostatin
Pilocarpine
10 Pirarubicin
Plicamycin
Porfimer Sodium
Procarbazine Hydrochloride
Raltitrexed
15 Romurtide
Sargramostim
Sizofilan
Sobuzoxane
Streptozocin 2-deoxy-2-((methylnitrosoamino)
20 carbonyl)amino)-alpha (and beta)-D-glucopyranose
Tamoxifen Citrate
Tegafur + uracil
TheraCys BCG Live
Thioguanine
25 Thiotepa
Topotecan
Topotecan Hydrochloride
Toremifene

Tretinoin
Vinblastine Hydrochloride
Vincristine Sulfate
Vinorelbine
5 Vinorelbine Tartrate
Zinostatin stimalamer
Ambamustine
Phenalon
Ukrain
10 Broxuridine
EF-13
EF-27
Emitefur
Liarozole
15 Mitoguazone
Pentostatin
Virulizin
Vorozole
9-aminocamptothecin
20 AC Vaccine Technology
AD-32
AG-337
ALRT-1057
Adenocarcinoma vaccine
25 Anti-Her-2 MAb
AS-101
Autolymphocyte therapy
CGP-19835A

Cancer therapy, Aquila Biopharmaceuticals
Crisnatol mesylate
Dexaminoglutethimide
Diaziquone
5 Droloxifene
Exemestane
FGN-1
Fenretinide
GMK
10 ICI-182780
JM-216
LGD-1069
Lisofylline
M-Vax
15 Marimastat
Maxamine
Neovastat
Onconase
PALA
20 Peldesine
Piritrexim
Porfiromycin
Regressin
SDZ-PSC-833
25 SnET2
Suramin
Temoporfin
Temozolomide

Tiazofurin
Tirapazamine
506U78
776C85
5 AGM-1470
ALRT-1550
Adenosine triphosphate
Alanosine
Aminopterin
10 Amrubicin
Annamycin
Anti-Bcl2 oligonucleotides
Antineoplaston A10
Antineoplaston AS2-1
15 BCH-4556
BEC-2
BMS-182248-01
BPA
Bisnafide
20 budotitane
CM-101
CTP-37
Calicheamicin
cancer vaccines, Wistar
25 Capecitabine
Carboxypeptidase
Carzelesin
cystemustine

DA-125
DHAC
DPPE
Decitabine
5 Didemnin B
Didox
EB-1089
EL-530
EL-532
10 EO9
ET-743
GBC-590
GL-331
Gd-TeX
15 HN-66000
HP-228
Homoharringtonine
IST-622
Idoxifene
20 Ifosfamide + methylene blue
Interleukin-3 synthokine
KRN-5500
KRN-8602
L-Vax
25 LY-231514
Ledoxantrone trihydrochloride
Lobaplatin
Lometrexol

Lu-Tex
MAK therapy
MAK-BAb
MGDF
5 MS-209
Melanoma vaccine
Metesind glucuronate
Miproxifene phosphate
NK-611
10 NKS01
Nemorubicin
Nitrullyn
NOAC
O-Vax
15 OC-TR
ONO-4007
POLYDAN
PPI-149
RF1010
20 RFS-2000
RII retinamide
RMP-7
Rhizoxin
S-1
25 SKI-2053R
SU101
Theradigm-melanoma
VX-710

VX-853
YM-511
42/6 Antibody
5-FP
5 AG-2034
AG-3340
Abiraterone acetate
BTG
Acemannan
10 Adenocarcinoma vaccine
Adenosine triphosphate
Alnorin
Antide
Aphidicolin glycinate
15 Asulacrine
BAB-447
BBR-2778
BCH-4556
BIWB-1
20 Bizelesin
Bryostatin-1
CEP-2563
CGP-41251
CGP-48664A
25 CGP-55847
CI-994
CT-2584
Cancer vaccine, Genzyme

	Clomesone
	Cordecypin
	Crisnatol mesylate
	Cyclocreatine
5	D-19575
	D-21266
	DX-8951f
	Diethylnorspermine
	Dolastatin-10
10	Edatrexate
	EM-800
	FCE-28068
	FK-317
	Flavopiridol
15	GF-120918
	Intoplicine
	KT-6149
	KW-2170
	KW-2189
20	LU-103793
	LU-79553
	LY-309887
	Lymphoma vaccine, Apollon
	MAC-DC
25	MDAM
	ME-2906
	Melanoma vaccine, UCLA
	MEN-10755

MGI-114
MGV
MKC-454
Methioninase
5 Muc-1 vaccine
NB-506
Norcantharidin
OGT-719
OM-174
10 Oligonucleotide AML
OncoLipin-2
PG-2
PR-350
Peptide G
15 Pivaloyloxymethyl butyrate
Quinocarmycin monocitrate
S-16020-2
SDZ-62-434
SDZ-MKT-077
20 TAS-103
Theophylline
TherAmide
Theratope MUC-1
Titanocene dichloride
25 Tularemia live vaccine
Tumour vaccines, Medac
UCN-01
XR-5000

ZD-9331

ZnPc

A-007

C215FAb-SEA

5

CAI

Dilazep, chemoprotective

Gossypol

HSP cancer vaccine

Neuropeptides, ICRT

10

Perillyl alcohol

Paracelsian

TOP-53

TZT-1027

15 Methods for the preparation of many of the
antineoplastic agents described above can be found in the
literature. For example, methods for the preparation of
doxorubicin are described in U.S. Patents Nos. 3,590,028
and 4,012,448. Alternatively, certain agents are available
commercially.

20

Pharmaceutical Compositions

 The iminosugar and chemotherapeutic compounds
employed in the methods of the present invention can be
administered for their therapeutic purposes by any means
that produce contact of these compounds with their site of
25 action either *in vitro* or *in vivo* within the body.

These compounds can be formulated separately, or together in a single pharmaceutical composition, along with a pharmaceutically acceptable carrier, diluent, or excipient. The carrier, etc., can be a solid, a liquid, or both, and is preferably formulated with the compound as a unit-dose composition, for example a tablet, which can contain from about 0.05% to about 95% by weight of the active compound(s). Other pharmacologically active substances can also be present. The pharmaceutical compositions of the present invention can be prepared by any of the well known techniques of pharmacy, consisting essentially of appropriately admixing the components. The formulation of pharmaceuticals is discussed in, for example, *Remington's Pharmaceutical Sciences*, 16th Edition, Arthur Osol, Ed., Mack Publishing Co., Easton, Pennsylvania (1980), and *Pharmaceutical Dosage Forms*, H.A. Liberman and L. Lachman, Eds., Marcel Decker, New York, N.Y. (1980).

The individual or combination pharmaceutical compositions of the present invention can be administered by any conventional means available for use in conjunction with pharmaceuticals. Pharmaceutical compositions according to the present invention include those suitable for oral, buccal (e.g., sublingual), parenteral (e.g., subcutaneous, intramuscular, intradermal, intrasternal, or intravenous injection, or infusion techniques), rectal, transdermal, and topical administration, as well as by inhalation spray, in dosage unit formulations containing

conventional nontoxic pharmaceutically acceptable carriers, adjuvants, and vehicles as desired. Topical administration can involve the use of transdermal administration such as transdermal patches or iontophoresis devices.

5 For therapeutic purposes, formulations for parenteral administration, for example sterile injectable aqueous or oleaginous suspensions, can be formulated according to the known art in the form of aqueous or non-
10 aqueous isotonic sterile injection solutions or suspensions using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic, parenterally acceptable diluent or solvent, for example as
15 a solution in 1,3-butanediol. These solutions and suspensions can be prepared from sterile powders or granules having one or more of the carriers or diluents mentioned for use in the formulations for oral
20 administration. Pharmaceutically acceptable vehicles for the compounds of the present invention include water, polyethylene glycol, propylene glycol, ethanol, corn oil, cottonseed oil, peanut oil, Ringer's solution, sesame oil, benzyl alcohol, isotonic sodium chloride solution, and/or
25 various buffers. In addition, sterile, fixed oils are conventionally employed as solvents or suspending media. For this purpose, any bland fixed oil can be employed, including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid are useful in the preparation of injectables. Other adjuvants and modes of

administration are well and widely known in the pharmaceutical art. Injectable compositions according to the present invention can contain from about 0.1% to about 5% w/w of a compound disclosed herein.

5 Solid dosage forms for oral administration may include capsules, cachets, lozenges, tablets, or pills, each containing a predetermined amount of at least one compound of the present invention, or as powders, and granules. In such solid dosage forms, the compounds of
10 this invention are ordinarily combined with one or more adjuvants appropriate to the indicated route of administration. If administered per os, the compounds can be admixed with lactose, sucrose, starch powder, cellulose esters of alkanolic acids, cellulose alkyl esters, talc,
15 stearic acid, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulfuric acids, gelatin, acacia gum, sodium alginate, polyvinylpyrrolidone, and/or polyvinyl alcohol, and then tableted or encapsulated for convenient administration. Such capsules or tablets
20 can contain a controlled-release formulation as can be provided in a dispersion of active compound in hydroxypropylmethyl cellulose. In the case of capsules, tablets, and pills, the dosage forms can also comprise buffering agents such as sodium citrate, or magnesium or
25 calcium carbonate or bicarbonate. Tablets and pills can additionally be prepared with enteric coatings.

Liquid dosage forms for oral administration can include pharmaceutically acceptable emulsions, solutions,

suspensions, syrups, and elixirs containing inert diluents commonly used in the art, such as water or other pharmaceutically acceptable non-aqueous liquid, or as an oil-in-water or water-in-oil emulsion. Such compositions can also comprise adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring, and perfuming agents.

Pharmaceutical compositions suitable for buccal (sub-lingual) administration include lozenges comprising a compound of the present invention in a flavored base, usually sucrose, and acacia or tragacanth, and pastilles comprising the compound in an inert base such as gelatin and glycerin or sucrose and acacia.

Unit-dose suppositories for rectal administration of the compounds discussed herein can be prepared by mixing the active agent with a suitable non-irritating excipient such as cocoa butter, synthetic mono-, di-, or triglycerides, fatty acids, or polyethylene glycols which are solid at ordinary temperatures but liquid at the rectal temperature, and which will therefore melt in the rectum and release the drug.

Pharmaceutical compositions suitable for topical application to the skin preferably take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers that can be used include vaseline, lanolin, polyethylene glycols, alcohols, and combinations of two or more thereof. The active compound can be present at a

concentration of from about 0.1% to about 15% w/w of the composition, for example, from about 0.5% to about 2%.

Transdermal administration is also possible. Pharmaceutical compositions suitable for transdermal administration can be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Such patches suitable contain a compound of the present invention in an optionally buffered, aqueous solution, dissolved and/or dispersed in an adhesive, or dispersed in a polymer. A suitable concentration of the active compound is in the range of from about 1% to about 35%, w/w, more preferably from about 3% to about 15%. As one particular possibility, the compound can be delivered from the patch by electrotransport or iontophoresis, for example, as described in *Pharmaceutical Research* (1986) 3:318.

Pharmaceutically acceptable carriers encompass all the foregoing and the like.

In addition to the foregoing types of pharmaceutical compositions, the iminosugars and chemotherapeutic compounds of the present invention can be administered in the form of delayed release or controlled release pharmaceutical preparations, i.e., pharmaceutical preparations designed to delay and/or extend the time over which the active drug molecule(s) is (are) delivered to the site of action by manipulation of the dosage form. In both cases, release of the pharmaceutically active agent is such that a pharmaceutically effective amount thereof capable of

achieving its intended effect is present *in vitro* or *in vivo* over an extended period of time. Encompassed within the scope of the present invention, therefore, are such preparations, wherein either drug is present separately, both drugs are present together, or wherein both drugs are present together in a single formulation, but wherein one or the other of the iminosugar or chemotherapeutic compound is present in delayed or controlled release form, and the other is not. Delayed and/or controlled release of the present iminosugar compounds is preferred due to their pharmacokinetic properties, i.e., the desirability of maintaining a constant blood serum level thereof over a prolonged period.

This can be achieved by a number of different mechanisms, including, for example, pH sensitive release from the dosage form based on the changing pH of the small intestine, slow erosion of a tablet or capsule, retention in the stomach based on the physical properties of the formulation, bioadhesion of the dosage form to the mucosal lining of the intestinal tract, enzymatic release of the active drug from the dosage form, etc. Delayed delivery dosage formulations are disclosed in U.S. Patent 5,190,765. Slow release pharmaceutical compositions are also well known in the art. For example, U.S. Patent No. 4,524,060 discloses a composition in the form of a non-compressed pellet having an enteric coat or a sustained release coat permeable to gastrointestinal juices. Other controlled

release formulations are described in U.S. Patents Nos. 4,880,830 and 5,068,112.

5 In addition to the delayed release and controlled release dosage formulations discussed above, there are dosage forms known in the art for delivering drugs continuously over time such as those disclosed in U.S. Patents Nos. 4,327,725, 4,612,008, 4,765,989, and 4,783,337 that comprise a semipermeable wall surrounding a compartment. The compartment contains a drug formulation and a displacement member that pushes the drug formulation from the dosage form when fluid is imbibed by the dosage form through the semipermeable wall. Such dosage forms can deliver difficult to deliver drugs for their intended purpose. Another type of controlled release drug formulation or device is the gliadel wafer (Guilford Pharmaceutical). This vehicle can be used for local administration, for example in a tumor bed, for example that in a brain tumor, of a chemotherapeutic agent such as BCNU.

20 In any case, the amount of active ingredient that can be combined with the carrier materials to produce a single dosage form to be administered will vary depending upon the patient, the nature of the formulation, and the mode of administration.

25 Certain of the pharmaceutical compounds of this invention which are administered in accordance with the methods of the invention can serve as prodrugs to other compounds of this invention. Prodrugs are drugs that can

be chemically converted *in vivo* or *in vitro* by biological systems into an active derivative or derivatives. Prodrugs are administered in essentially the same fashion as the other pharmaceutical compounds of the invention. Non-limiting examples are the esters of the *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds of this invention.

It should be noted that the pharmaceutical compositions of the present invention can contain individual iminosugars, or combinations thereof, in anti-MDR effective doses. These iminosugars can also be used in combination with anti-MDR effective amounts of other compounds useful as anti-MDR agents, such as verapamil, tamoxifen, cyclosporin A, etc. In addition, the present invention encompasses pharmaceutical compositions comprising at least one of the present *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds and at least one anti-tumor chemotherapeutic compound. In such combined compositions, the iminosugar should be present in an anti-MDR effective amount, and the anti-tumor chemotherapeutic compound should be present in an anti-tumor effective amount. Specific dosages are discussed in detail below.

Administration

The *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds and one or more

antineoplastic agents can be administered either sequentially in separate formulations, or simultaneously in a single formulation. Either the iminosugar or the antineoplastic agent, or both, can be used in combination with a liposome formulation to deliver the iminosugar and/or antineoplastic agent to the target tumor while protecting more sensitive tissue from the toxic effect of the antineoplastic agent. Administration can be effected by the route appropriate to the formulation of the pharmaceutical composition, discussed above. Administration by oral route is preferred in the case of the present iminosugars, but other routes are acceptable. Administration of anti-neoplastic chemotherapeutic agents can be by any conventional route therefor, which includes oral route, or intravenous, intra-muscular, or subcutaneous injection or infusion. Administration of pharmaceutical compositions comprising both an iminosugar and an antineoplastic chemotherapeutic agent can thus be performed by any acceptable route compatible with both classes of compounds contained therein, such as the latter routes. Combination formulations can be in the form of a bolus, or in the form of aqueous or non-aqueous isotonic sterile injection solutions or suspensions. These solutions and suspensions can be prepared from sterile powders or granules having one or more pharmaceutically acceptable carriers, excipients, or diluents, or a binder such as gelatin or hydroxypropyl-methyl cellulose, together with

one or more of a lubricant, preservative, surface-active agent, or dispersing agent.

Dosages

Imino Sugars

5 To prevent, reduce, or reverse MDR during chemotherapy, the *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol and/or galactitol compounds of the present invention should be administered to humans, or domestic animals such as cats and dogs, in an anti-MDR effective amount. Functionally, an effective amount is an amount, by
10 whatever route administered, that results in a blood serum concentration in the range of from about 5 μ M to about 500 μ M, preferably from about 10 μ M to about 250 μ M, more preferably from about 15 μ M to about 100 μ M, and even more
15 preferably from about 20 μ M to about 60 μ M. About 50 μ M is a preferred concentration. This can be achieved by administration of these compounds in an amount in the range of from about 10 mg/day to about 3,000 mg/day, more preferably from about 100 mg/day to about 3,000 mg/day, and
20 most preferably from about 1,000 mg/day to about 3,000 mg/day. About 3,000 mg/day is a preferred dose. When administered in non-sustained release formulations, the total daily dose of iminosugars indicated above can be administered in equal, one-third subdoses administered at
25 eight hour intervals, e.g., about 1,000 mg every eight hours. When a sustained-release preparation is employed, the total daily dose can be administered at one time. In

either case, the pharmaceutical composition should contain an amount of iminosugar effective to achieve a blood serum level in the micromolar ranges indicated above over successive 8 hour intervals.

5 In a 24 week study of the safety and efficacy of N-butyl DNJ and zidovudine in patients with HIV-1 infection, Fischl et al. ((1994) *J. Acquired Immune Defic. Syndr.* 7:139) noted that the major toxicity associated with administration of 3,000 mg/day of N-butyl DNJ was diarrhea.
10 These authors suggested that such diarrhea could be alleviated with a low complex carbohydrate diet and/or antidiarrheal medications.

 N-alkylated glucitol and galactitol iminosugars each possess distinct advantages in the methods of the present invention. N-butyl DNJ does not inhibit the
15 galactosyltransferase that initiates the biosynthesis of galactosylceramide (GalCer)-based glycosphingolipids (GalCer and sulfatide), which are important constituents of myelin. Thus, N-butyl DNJ and related glucitol derivatives
20 will not impair myelination and myelin stability in patients in which this is a concern.

 On the other hand, in patients in which inhibition of α -glucosidase I and II or lysosomal β -glucocerebrosidase is undesirable, N-alkyl galactitol
25 iminosugars may be preferred in view of the specificity of compounds such as N-butyl DGJ in inhibiting

glycosphingolipid biosynthesis (Platt et al. (1994) *J. Biol. Chem.* 269:27108-27114).

In some situations, it may be desirable to use a pharmaceutical composition comprising a combination of an
5 *N*-alkyl glucitol and an *N*-alkyl galactitol iminosugar to avoid or ameliorate the effects of MDR during chemotherapy. Together, such iminosugars should comprise an anti-MDR effective amount.

Chemotherapeutic Agents

10 Guidelines for drug selection and dosage for the treatment of cancer can be found in *Cancer: Principles & Practice of Oncology*, 6th Edition, 1996, Vincent T. DeVita, Jr. et al., Eds., J.B. Lippincott Company, Philadelphia.

Due to suppression of MDR via the use of the
15 *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds of the present invention, the medical practitioner will be able to administer conventional amounts of chemotherapeutic agents, or perhaps even reduced amounts thereof, by employing the methods and compositions
20 disclosed herein. Such reduced amounts can be determined in patients undergoing chemotherapy by routine monitoring of tumor antigens, such as the CEA, PSA, or CA15-3 antigens, in patient serum, or in body tissues by other immunological methods; X-ray studies; radiographic imaging
25 of tumors; CT, MRI, ultrasound, or PET scanning; biopsy; palpation; observation of the general state of the patient,

performance status, etc., as is well known in the art. Thus, patients can be monitored during chemotherapy in conjunction with the administration of *N*-substituted- 1,5-dideoxy-1,5-imino-D-glucitol and/or galactitol compounds and antineoplastic agents to determine the lowest effective doses of each.

The doses described above can be administered to a patient in a single dose or in proportionate multiple subdoses. In the latter case, dosage unit compositions can contain such amounts of submultiples thereof to make up the total dose. Multiple subdoses can also be administered to increase the total dose should this be desired by the person prescribing the drug.

Combination Pharmaceutical Compositions

As noted above under "Pharmaceutical Compositions," the iminosugar and chemotherapeutic compounds employed in the methods of the present invention can be formulated in single pharmaceutical compositions comprising both classes of drugs. Such compositions should contain an iminosugar in an anti-MDR effective dosage amount and an anti-tumor chemotherapeutic compound in an anti-tumor effective dosage amount. An anti-MDR effective dosage amount of an iminosugar is an amount, by whatever route administered, that results in a blood serum concentration in the range of from about 5 μ M to about 500 μ M, preferably from about 10 μ M to about 250 μ M, more preferably from about 15 μ M to about 100 μ M, and even more

preferably from about 20 μ M to about 60 μ M. About 50 μ M is a preferred concentration. When administered in a delayed or controlled release formulation, this can be achieved by administration of these compounds in an amount in the range of from about 10 mg/day to about 3,000 mg/day, more preferably from about 100 mg/day to about 3,000 mg/day, and most preferably from about 1,000 mg/day to about 3,000 mg/day. About 3,000 mg/day is a preferred dose. Non-controlled release formulations should contain one-third of the total daily dose, e.g., about 1,000 mg, and should be administered to the patient at eight hour intervals.

Dosages for antineoplastic agents are described in *Cancer: Principles & Practice of Oncology*, 6th Edition, 1996, Vincent T. DeVita, Jr. et al., Eds., J.B. Lippincott Company, Philadelphia, or are otherwise known in the art. When administered in a delayed or controlled release form combination formulation containing an iminosugar, both the antineoplastic agent and the iminosugar can be administered in their standard daily, single administration dose. When administered in a combination formulation containing an iminosugar in non-controlled release form, the antineoplastic agent can be present in an amount totalling one-third of the total daily dose; such non-sustained release combination formulations should be administered to the patient at eight hour intervals to achieve the desired, total daily doses of both drugs. Alternatively, when an appropriate antineoplastic agent is given, the total daily dose of such antineoplastic agent can be present in

controlled or non-controlled release form for once daily administration, and the iminosugar can be present in non-controlled release form equivalent to one-third of the total daily dose, the two remaining one-third daily subdoses of the iminosugar being administered at subsequent eight hour intervals during the remainder of the day.

Treatment Regimen

The regimen for treating a patient undergoing chemotherapy with the compounds and/or compositions of the present invention is selected in accordance with a variety of factors, including the age, weight, sex, diet, and medical condition of the patient, the severity of the cancer, the route of administration, pharmacological considerations such as the activity, efficacy, pharmacokinetic, and toxicology profiles of the particular compounds employed, and whether a drug delivery system is utilized.

Typical chemotherapeutic regimens comprise a course of six to eight cycles of treatment, each cycle typically involving administration of antineoplastic drugs over the course of three to four weeks.

The N-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds of the present invention can be administered daily to patients receiving chemotherapy in accordance with a number of different regimens. Fundamentally, these iminosugars should be administered in an anti-MDR effective amount for a period

of time effective to exert their MDR preventing, reducing, or reversing action on tumor cells. Without wishing to be bound by any particular theory of this invention, the inventor hypothesizes that this effect may be achieved by inhibition of UDP-glucose-*N*-acyl-sphingosine glucosyltransferase (EC 2.4.1.80) for a period of time sufficient to decrease the levels of glucosylceramide, and subsequently, more complex glycosphingolipids and gangliosides, in the membranes of cancerous cells. Based upon results obtained in *in vitro* systems and Tay-Sachs mice, administration can commence in a period in the range of from about 14 days to about three days prior to administration of the chemotherapeutic agent(s), and can continue daily thereafter, up to and including administration of the chemotherapeutic agent. Administration of these iminosugars can be continued daily for a brief period, e.g., about one to about five days after administration of the chemotherapeutic agent, to alleviate or avoid potential MDR effects during the period in which residual amounts of chemotherapeutic agents remain in tumor cells.

Therefore, in general, the *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds of the present invention can be administered prior to administration of the chemotherapeutic agent. The *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds can also be administered both prior to

and simultaneously with administration of the
chemotherapeutic agent; or simultaneously with
administration of the chemotherapeutic agent; or prior to,
simultaneously with, and subsequently to administration of
the chemotherapeutic agent; or prior to and subsequently to
administration of the chemotherapeutic agent.

More particularly, the present N-substituted-1,5-
dideoxy-1,5-imino-D-glucitol or galactitol compounds can be
administered daily to the patient in a time period starting
from about 14 days prior to administration of the
chemotherapeutic agent. More preferably, these iminosugars
can be administered daily to the patient in a time period
starting from about 10 days prior to administration of the
chemotherapeutic agent. In some patients, it may be
necessary or desirable to commence administration of these
iminosugars about 7 days prior to administration of the
chemotherapeutic agent. In other cases, administration of
these iminosugars can commence about 5 days, or even about
3 days, prior to administration of the chemotherapeutic
agent. As indicated above, these iminosugars can be
further administered simultaneously with the
chemotherapeutic agent, and/or subsequently to
administration of the chemotherapeutic agent, on a daily
basis for a period in the range of from about one to about
five days, preferably for about two days, after
administration of each dose of the chemotherapeutic agent.

Administration of the *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds of the present invention should be continued in conjunction with the prescribed chemotherapeutic regimen as outlined above until the cancer has been controlled or eradicated.

The proven long-term safety associated with the administration of the iminosugars disclosed herein (note, for example, Fischl et al. ((1994) *J. Acquired Immune Defic. Syndr.* 7:139, in this regard) also permits another regimen: the present *N*-alkylated glucitol and galactitol derivatives can be administered on a daily basis throughout the entire course of the patient's chemotherapy. Rather than administering these compounds only in anticipation of individual chemotherapy sessions as described above, the practitioner can order continuous daily administration thereof. In this regimen, and in a manner similar to that of the regimens described above, administration of the present *N*-alkylated glucitol and galactitol derivatives can commence about 14 days, about 10 days, about 7 days, about 5 days, or about 3 days prior to administration of the initial dose of the chemotherapeutic drug, and continue on a daily basis thereafter.

As previously noted, patients undergoing treatment with the drug combinations disclosed herein can be routinely monitored by measuring serum antigen levels, by radiographic imaging of tumors, biopsy, palpation, etc., to determine the effectiveness of therapy.

Continuous analysis of the data obtained by the foregoing methods permits modification of the treatment regimen during chemotherapy so that optimal amounts of the *N*-alkyl-1,5-dideoxy-1,5-imino-D-glucitol and galactitol compounds of this invention and chemotherapeutic agent(s) are administered, and so that the duration of treatment can be determined as well. Thus, the treatment regimen/dosing schedule can be rationally modified over the course of chemotherapy so as to achieve the lowest doses of each of the *N*-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compounds of this invention and the chemotherapeutic agent(s), which together result in satisfactory anti-cancer effectiveness, and so that administration of these compounds is continued only so long as is necessary to successfully treat the cancer.

The following non-limiting examples serve to illustrate various aspects of the present invention.

Example 1

Preparation of

1,5-(butylimino)-1,5-dideoxy-D-glucitol

A solution of 1,5-dideoxy-1,5-imino-D-glucitol (5.14 g, 0.0315 mole), butyraldehyde (3.35 ml, 0.0380 mole) and Pd black (1 g) in 200 ml methanol is hydrogenated (60 psi/29°C/21 hrs.). After filtering the resulting mixture, the filtrate is concentrated in vacuo to an oil. The title compound is crystallized from acetone, and recrystallized from methanol/acetone, m.p. ca. 132°C. The structure

assignment is supported by NMR, infrared spectra and elemental analysis.

Analysis calcd. for $C_{10}H_{21}NO_4$: C, 54.78; H, 9.65; N, 6.39. Found: C, 54.46; H, 9.33; N, 6.46.

Example 2

Preparation of

1,5-(butylimino)-1,5-dideoxy-D-glucitol, tetraacetate

Acetic anhydride (1.08 g, 0.0106 mole) is added to the title compound of Example 1 (0.50 g, 0.0023 mole) in 5 ml pyridine and stirred for 17 days at room temperature. The product is evaporated under nitrogen gas. The resulting title compound is purified by silica gel chromatography. The structure assignment is supported by NMR, infrared spectra, and elemental analysis.

Analysis calcd. for $C_{11}H_{23}NO_8$: C, 55.80; H, 7.54; N, 3.62. Found: C, 55.42; H, 7.50; N, 3.72.

Example 3

Preparation of

1,5-(butylimino)-1,5-dideoxy-D-galactitol

30 mg (184 μ mol) of deoxygalactonojirimycin are dissolved in 1 ml of 50 mM sodium acetate buffer, pH 5.0, to which 20 mg of palladium black is added. A hydrogen atmosphere is maintained in the reaction vessel, and 100 μ l (1.1 mmol) of butyraldehyde are introduced. The reaction is stirred for 16 hr. at room temperature (ca. 20°C). The

reaction is stopped by filtration through a bed (1 g) of Celite (30-80 mesh), and the reaction products are separated by chromatography using a column containing 4 ml of packed Dowex® AG50-X12 (H⁺ form) resin. The *N*-butyl DGJ is eluted from the column with 2M ammonia, and its molecular mass and chemical structure determined by laser desorption mass spectrometry and 1D ¹H NMR, respectively.

Example 4

Preparation of

1,5-(propylimino)-1,5-dideoxy-D-galactitol

The synthetic procedure and compound analysis of Example 3 can be repeated, except that propanoyl aldehyde can be substituted for an equivalent amount of butyraldehyde for analogous preparation of *N*-propyl DGJ.

Example 5

Preparation of

1,5-(hexylimino)-1,5-dideoxy-D-galactitol

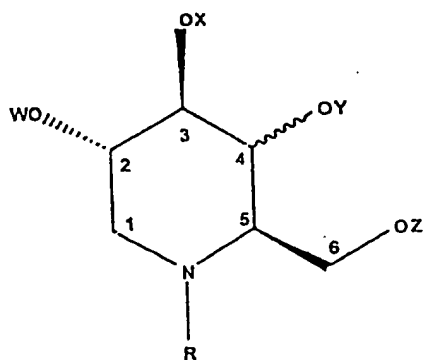
The synthetic procedure and compound analysis of Example 3 can be repeated, except that caproaldehyde can be substituted for an equivalent amount of butyraldehyde for analogous preparation of *N*-hexyl DGJ.

N-alkyl DGJ compounds prepared as described in foregoing Examples 3-5 can be obtained in overall yields of 68-74% based on the starting DGJ, and in greater than 95% purity.

The invention being thus described, it will be obvious that the same can be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the present invention, and all such modifications and equivalents as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

CLAIMS

1. A compound for preventing, reducing, or reversing multidrug resistance in a patient undergoing treatment with a chemotherapeutic agent, comprising an anti-multidrug resistance effective amount of an N-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compound, or a pharmaceutically acceptable salt thereof, of Formula I:



wherein R is selected from the group consisting of arylalkyl, cycloalkylalkyl, and branched or straight chain alkyl having a chain length of C₂ to C₂₀, and

W, X, Y and Z are independently selected from the group consisting of hydrogen, alkanoyl, aroyl, and trifluoroalkanoyl.

2. The compound of claim 1, wherein R is a straight or branched chain alkyl group having a chain length of C₂ to C₂₀, and W, X, Y, and Z are each hydrogen.

3. The compound of claim 2, wherein R is a straight chain alkyl group having a chain length of C₄ to C₂₀.

4. The compound of claim 3, wherein R is a straight chain alkyl group having a chain length of C₄ to C₁₄.

5. The compound of claim 4, wherein R is a straight chain alkyl group having a chain length of C₄ to C₁₀.

6. The compound of claim 5, wherein R is a straight chain alkyl group having a chain length of C₄ to C₈.

7. The compound of claim 6, wherein R is a straight chain alkyl group having a chain length of C₄ to C₆.

8. The compound of claim 7, wherein R is n-butyl.

9. The compound of claim 7, wherein R is n-hexyl.

10. The compound of claim 1, wherein R is a straight or branched chain alkyl group having a chain length of C₂ to C₂₀, and W, X, Y, and Z are each an alkanoyl group having a chain length of C₁ to C₂₀.

11. The compound of claim 10, wherein R is a straight chain alkyl group having a chain length of C₄ to C₂₀.

12. The compound of claim 1, wherein said N-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compound is selected from the group consisting of:

N-(n-ethyl-)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;

N-(n-propyl-)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;

N-(n-butyl-)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol;

- N- (n-hexyl-) -1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- N- (n-heptyl-) -1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- 5 N- (n-octyl-) -1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- N- (n-octyl-) -1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrates;
- N- (n-nonyl-) -1,5-dideoxy-1,5-imino-D-glucitol or
10 galactitol, tetrabutyrates;
- N- (n-decyl-) -1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrates;
- N- (n-undecyl-) -1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrates;
- 15 N- (n-nonyl-) -1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- N- (n-decyl-) -1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- N- (n-undecyl-) -1,5-dideoxy-1,5-imino-D-glucitol or
20 galactitol;
- N- (n-dodecyl-) -1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- N- (2-ethylhexyl) -1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- 25 N- (4-ethylhexyl) -1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- N- (5-methylhexyl) -1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
- N- (3-propylhexyl) -1,5-dideoxy-1,5-imino-D-glucitol or
30 galactitol;
- N- (1-pentylpentylhexyl) -1,5-dideoxy-1,5-imino-D-glucitol or

- galactitol;
N-(1-butylbutyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
N-(7-methyloctyl)-1,5-dideoxy-1,5-imino-D-glucitol or
5 galactitol;
N-(8-methylnonyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
N-(9-methyldecyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
10 N-(10-methylundecyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
N-(6-cyclohexylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
N-(4-cyclohexylbutyl)-1,5-dideoxy-1,5-imino-D-glucitol or
15 galactitol;
N-(2-cyclohexylethyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
N-(1-cyclohexylmethyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
20 N-(1-phenylmethyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
N-(3-phenylpropyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
N-(3-(4-methyl)-phenylpropyl)-1,5-dideoxy-1,5-imino-D- or
25 glucitol galactitol;
N-(6-phenylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol;
N-(n-nonyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrates;
30 N-(n-decyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrates;

- N-(n-undecyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N-(n-dodecyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- 5 N-(2-ethylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N-(4-ethylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N-(5-methylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or
10 galactitol, tetrabutyrate;
- N-(3-propylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N-(1-pentylpentylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- 15 N-(1-butylbutyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N-(7-methyloctyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N-(8-methylnonyl)-1,5-dideoxy-1,5-imino-D-glucitol or
20 galactitol, tetrabutyrate;
- N-(9-methyldecyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N-(10-methylundecyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- 25 N-(6-cyclohexylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N-(4-cyclohexylbutyl)-1,5-dideoxy-1,5-imino-D-glucitol or
galactitol, tetrabutyrate;
- N-(2-cyclohexylethyl)-1,5-dideoxy-1,5-imino-D-glucitol or
30 galactitol, tetrabutyrate;
- N-(1-cyclohexylmethyl)-1,5-dideoxy-1,5-imino-D-glucitol or

galactitol, tetrabutyrates;

N-(1-phenylmethyl)-1,5-dideoxy-1,5-imino-D-glucitol or

galactitol, tetrabutyrates;

N-(3-phenylpropyl)-1,5-dideoxy-1,5-imino-D-glucitol or

5 galactitol, tetrabutyrates;

N-(3-(4-methyl)-phenylpropyl)-1,5-dideoxy-1,5-imino-D-

glucitol or galactitol, tetrabutyrates; and

N-(6-phenylhexyl)-1,5-dideoxy-1,5-imino-D-glucitol or

galactitol, tetrabutyrates, or

10 a pharmaceutically acceptable salt thereof.

13. The compound of claim 12, wherein said N-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compound is selected from the group consisting of N-(n-butyl)-1,5-dideoxy-1,5-imino-D-glucitol or

15 galactitol and N-(n-hexyl)-1,5-dideoxy-1,5-imino-D-glucitol or galactitol.

14. The compound of claim 1, wherein said chemotherapeutic agent is selected from the group consisting of an alkaloid, a topoisomerase II inhibitor,

20 and a DNA damaging agent.

15. The compound of claim 14, wherein said alkaloid is a vinca alkaloid.

16. The compound of claim 15, wherein said vinca alkaloid is selected from the group consisting of

25 vincristine, vinblastine, and vindesine.

17. The compound of claim 14, wherein said topoisomerase II inhibitor is selected from the group consisting of an anthracycline and an epipodophyllotoxin.

18. The compound of claim 17, wherein said

30 anthracycline is selected from the group consisting of doxorubicin, daunorubicin, idarubicin, and mitoxantrone.

19. The compound of claim 17, wherein said epipodophyllotoxin is selected from the group consisting of etoposide and tenoposide.

5 20. The compound of claim 14, wherein said DNA damaging agent is actinomycin D.

21. The compound of claim 1, wherein said effective amount of said N-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compound is an amount that results in a blood serum concentration in the range of from about 10 5 μ M to about 500 μ M by whatever route it is administered.

22. The compound of claim 21, wherein said effective amount of said N-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compound is an amount that results in a blood serum concentration in the range of 15 from about 20 μ M to about 60 μ M by whatever route it is administered.

23. The compound of claim 22, wherein said effective amount of said N-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compound is an amount that results in a blood serum concentration of about 50 μ M by 20 whatever route it is administered.

24. A pharmaceutical composition, comprising an anti-multidrug resistance effective amount of at least 25 one N-substituted-1,5-dideoxy-1,5-imino-D-glucitol or galactitol compound of claim 1;

an anti-tumor effective amount of at least one anti-tumor chemotherapeutic compound; and

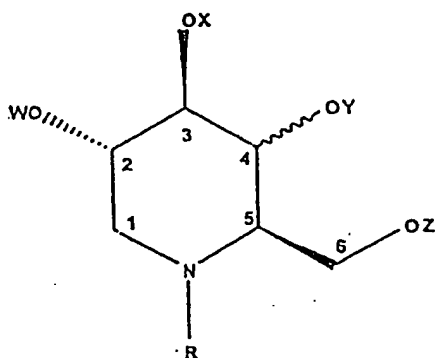
a pharmaceutically acceptable carrier.

30 25. The pharmaceutical composition of claim 24, wherein both said N-substituted-1,5-dideoxy-1,5-imino-D-

glucitol or galactitol compound and said anti-tumor
chemotherapeutic compound are in controlled release form.

26. The pharmaceutical composition of claim 24,
wherein only said N-substituted-1,5-dideoxy-1,5-imino-D-
glucitol or galactitol compound is in controlled release
form.

27. The use of an N-substituted-1,5-dideoxy-1,5-
imino-D-glucitol or galactitol compound, or a
pharmaceutically acceptable salt thereof, of Formula I:



I

wherein R is selected from the group consisting of
arylalkyl, cycloalkylalkyl, and branched or straight
chain alkyl having a chain length of C₂ to C₂₀, and

W, X, Y and Z are independently selected from the
group consisting of hydrogen, alkanoyl, aroyl, and
trifluoroalkanoyl, for manufacture of a medicament for
preventing, reducing, or reversing multidrug resistance
in a patient undergoing treatment with a chemotherapeutic
agent.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/23239

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07D211/46 A61K31/445

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 566 556 A (G.D. SEARLE & CO.) 20 October 1993 see page 2 - page 3 ---	1, 24
A	EP 0 494 850 A (G.D. SEARLE & CO.) 15 July 1992 see the whole document ---	1, 24
A	EP 0 324 328 A (MONSANTO COMPANY) 19 July 1989 see the whole document & US 4 849 430 A cited in the application ---	1, 24
A	WO 95 22975 A (G. D. SEARLE & CO.) 31 August 1995 see page 2 - page 5 ---	1, 24
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

S document member of the same patent family

Date of the actual completion of the international search

31 March 1999

Date of mailing of the international search report

09/04/1999

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/23239

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 2 020 278 A (NIPPON SHINYAKU) 14 November 1979 see the whole document & US 4 639 436 A cited in the application -----	1,24
A	US 5 536 732 A (BRIGITTE LESUR ET AL.) 16 July 1996 see the whole document -----	1,24

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/23239

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 566556 A	20-10-1993	US 5258518 A	02-11-1993
		AT 139998 T	15-07-1996
		CA 2093078 A	02-10-1993
		DE 69303413 D	08-08-1996
		DK 566556 T	19-08-1996
		ES 2090948 T	16-10-1996
		GR 3021228 T	31-01-1997
		JP 6279408 A	04-10-1994
		US 5350854 A	27-09-1994
		US 5530132 A	25-06-1996
		US 5523406 A	04-06-1996
		US 5502193 A	26-03-1996
		US 5637707 A	10-06-1997
		US 5639882 A	17-06-1997
EP 494850 A	15-07-1992	US 5144037 A	01-09-1992
		CA 2059063 A	11-07-1992
		JP 4334368 A	20-11-1992
		US 5221746 A	22-06-1993
EP 324328 A	19-07-1989	US 4849430 A	18-07-1989
		AT 159427 T	15-11-1997
		AU 605661 B	17-01-1991
		AU 2706788 A	22-06-1989
		CA 1316459 A	20-04-1993
		DE 3856052 D	27-11-1997
		DE 3856052 T	19-02-1998
		DK 707988 A	22-06-1989
		ES 2108680 T	01-01-1998
		GR 3025518 T	27-02-1998
		JP 1203326 A	16-08-1989
		JP 1810437 C	27-12-1993
		JP 5021891 B	25-03-1993
		NZ 227411 A	24-03-1997
		OA 9027 A	31-03-1991
		PT 89266 A,B	29-12-1989
WO 9522975 A	31-08-1995	AU 1876095 A	11-09-1995
		US 5622972 A	22-04-1997
GB 2020278 A	14-11-1979	JP 1301674 C	14-02-1986
		JP 55098163 A	25-07-1980
		JP 60026387 B	24-06-1985
		JP 1266534 C	27-05-1985
		JP 54145672 A	14-11-1979
		JP 59043947 B	25-10-1984
		JP 1266536 C	27-05-1985
		JP 55009051 A	22-01-1980
		JP 59043948 B	25-10-1984
		JP 1266539 C	27-05-1985
		JP 55047655 A	04-04-1980
		JP 59043949 B	25-10-1984
		AT 371441 B	27-06-1983
		AT 278781 A	15-11-1982
		AT 324779 A	15-11-1982
		CH 642629 A	30-04-1984
		DE 2915037 A	08-11-1979
		DK 178379 A,B,	04-11-1979

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/23239

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2020278 A		FR 2424910 A	30-11-1979
		NL 7903421 A,B,	06-11-1979
		SE 436874 B	28-01-1985
		SE 7903817 A	04-11-1979
		SE 451015 B	24-08-1987
		SE 8402549 A	11-05-1984
		SE 451016 B	24-08-1987
		SE 8402550 A	11-05-1984
		SE 451017 B	24-08-1987
		SE 8402551 A	11-05-1984
		US 4533668 A	06-08-1985
		AT 371439 B	27-06-1983
		BE 876020 A	03-09-1979
		AT 371440 B	27-06-1983
		AT 278581 A	15-11-1982
		AT 372945 B	25-11-1983
		AT 278681 A	15-04-1983
US 5536732 A	16-07-1996	EP 0453692 A	30-10-1991
		AT 132153 T	15-01-1996
		AU 632926 B	14-01-1993
		AU 7535191 A	07-11-1991
		CA 2041331 A	28-10-1991
		CN 1056104 A,B	13-11-1991
		DE 69115750 D	08-02-1996
		DE 69115750 T	15-05-1996
		DK 454580 T	29-01-1996
		EP 0454580 A	30-10-1991
		ES 2084126 T	01-05-1996
		FI 912033 A,B,	28-10-1991
		GR 3018797 T	30-04-1996
		HU 212496 B	29-07-1996
		IE 72477 B	23-04-1997
		IL 97975 A	14-05-1996
		JP 5086072 A	06-04-1993
		NO 179913 B	30-09-1996
		PT 97485 A,B	31-01-1992
		US 5252587 A	12-10-1993

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<p>(21) International Application Number: PCT/US99/27918 (22) International Filing Date: 8 December 1999 (08.12.99) (30) Priority Data: 60/111,683 10 December 1998 (10.12.98) US (71) Applicants (for all designated States except US): G.D. SEARLE & CO. [US/US]; Corporate Patent Department, P.O. Box 5110, Chicago, IL 60680-5110 (US). UNIVERSITY OF OXFORD [GB/GB]; South Parks Road, Oxford OX1 3QU (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): JACOB, Gary, S. [US/US]; 12541 Mason Forest Drive, St. Louis, MO 63141 (US). PLATT, Frances, M. [GB/GB]; Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU (GB). BUTTERS, Terry, D. [GB/GB]; Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU (GB). DWEK, Raymond, A. [GB/GB]; Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU (GB).</p>		<p>(74) Agents: WILLIAMS, Roger, A. et al.; G.D. Searle & Co., Corporate Patent Department, P.O. Box 5110, Chicago, IL 60680-5110 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: USE OF LONG-CHAIN N-ALKYL DERIVATIVES OF DEOXYNOJIRIMYCIN FOR THE MANUFACTURE OF A MEDICAMENT FOR THE TREATMENT OF GLYCOLIPID STORAGE DISEASES (57) Abstract <p>A novel method is disclosed for the treatment of a patient affected with Gaucher's disease or other such glycolipid storage diseases. The method comprises administering to said patient a therapeutically effective amount of a long-chain N-alkyl derivative of deoxynojirimycin to alleviate or inhibit the glycolipid storage disease. The long-chain alkyl group has from nine to about 20 carbon atoms and preferably is nonyl or decyl.</p></p>		

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USE OF LONG-CHAIN N-ALKYL DERIVATIVES OF DEOXYNOJIRIMYCIN FOR THE MANUFACTURE OF A
MEDICAMENT FOR THE TREATMENT OF GLYCOLIPID STORAGE DISEASES

BACKGROUND OF THE INVENTION

The present invention relates to a method for the treatment of Gaucher's disease and other glycolipid storage diseases.

Gaucher's disease is a glycolytic storage disease caused by a genetic deficiency in activity of the catabolic enzyme beta-glucocerebrosidase. Beutler, Proc. Natl. Acad. Sci. USA 90, 5384-5390 (1993). Manifestations of this disease are impaired hematopoiesis, bone fractures, a thinning of the bone cortex and massive enlargement of the spleen and liver.

In recent years, several therapies have been proposed for the treatment of Gaucher's disease. An early therapeutic approach involved replacement of the deficient enzyme. See, for example, Dale and Beutler, Proc. Natl. Acad. Sci. USA 73, 4672-4674 (1976); Beutler et al., Blood 78, 1183-1189 (1991); and Beutler, Science 256, 794-799 (1992).

Leading commercial products for enzyme replacement are CEREDASE (glucocerebrosidase), which is derived from human placental tissues, and CEREZYME (recombinant human glucocerebrosidase), both of which are produced by Genzyme Corp.

See, for example, U.S. Patent Nos. 3,910,822; 5,236,838; and 5,549,892.

Conjugates of the glucocerebrosidase enzyme with polyethylene glycol (PEG) have also been advanced by Enzon Inc. for treatment of Gaucher's disease. See, for example, U.S. Patent Nos. 5,705,153 and 5,620,884.

Still another approach for treatment of the disease is gene therapy, which involves an *ex vivo* gene transfer protocol.

Another recent approach involves administration of the totally synthetic drugs, N-butyldeoxynojirimycin and N-butyldeoxygalactonojirimycin, as described, respectively, by Platt et al., J. Biol. Chem. **269**, 8362-8365 (1994); Id. **269**, 27108-27114 (1994). See also, U.S. Patent Nos. 5,472,969; 5,786,368; 5,798,366; and 5,801,185.

N-butyldeoxynojirimycin (N-butyl-DNJ) and related N-alkyl derivatives of DNJ are known inhibitors of the N-linked oligosaccharide processing enzymes, α -glucosidase I and II. Saunier et al., J. Biol. Chem. **257**, 14155-14161 (1982); Elbein, Ann. Rev. Biochem. **56**, 497-534 (1987). As glucose analogs, they also have potential to inhibit glycosyltransferases. Newbrun et al., Arch. Oral Biol. **28**, 516-536 (1983); Wang et al., Tetrahedron Lett. **34**, 403-406 (1993). Their inhibitory activity against the glycosidases has led to the development of these compounds as antihyperglycemic agents and as antiviral agents. See, e.g., PCT Int'l. Appln. WO 87/030903 and U.S. Patent Nos. 4,065,562; 4,182,767; 4,533,668; 4,639,436; 5,011,829; and 5,030,638.

In particular, N-butyl-DNJ has been developed as an inhibitor of human immunodeficiency virus (HIV) as described by Karpas et al., Proc. Nat'l. Acad. Sci. USA **85**, 9229-9233 (1988), U.S. Patent 4,849,430; and as an inhibitor of hepatitis B virus (HBV) as described by Block et al., Proc. Natl. Acad. Sci. USA **91**, 2235-2239 (1994), PCT Int'l. Appln. WO 95/19172.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, a novel method is provided for the treatment of a patient affected with Gaucher's disease or other such glycolipid storage diseases. The method comprises administering to said patient a therapeutically effective amount of a long-chain N-alkyl derivative of 1,5-dideoxy-1,5-imino-D-glucitol having from nine to about 20 carbon atoms in the alkyl chain. The N-alkyl substituent thus can be, e.g, nonyl, decyl, undecyl, dodecyl, tetradecyl, hexadecyl, cis-11-hexadecenyl, octadecyl, cis-13-octadecenyl, and eicosyl. A therapeutically effective amount is meant an amount effective in alleviating or inhibiting Gaucher's disease or other such glycolipid storage diseases in said patient.

The alkyl group in these long-chain N-alkyl-DNJ compounds preferably contains nine to ten carbon atoms (i.e., nonyl and decyl). A most preferred compound is N-nonyl-1,5-dideoxy-1,5-imino-D-glucitol, also known as the N-nonyl derivative of deoxynojirimycin (DNJ), which also is abbreviated herein as N-nonyl-DNJ.

In the field of general organic chemistry, the long-chain alkyl groups are known to provide more hydrophobic properties to compounds than are the short-chain alkyl groups. That is, solubility with water decreases with increase in chain length and

decrease in temperature. For example, at 46°C, caproic acid (short-chain hexyl group) dissolves 10% by weight of water, whereas stearic acid (long-chain octadecyl group) dissolves only 0.92% even at the higher temperature of 69°C. Bailey's Industrial Oil and Fat Products, ed. Daniel Swern, 3d ed. 1964, p. 126.

The long-chain N-alkyl derivatives of DNJ are known amino-sugar compounds. They were originally described as members of a group of short-chain and long-chain N-alkyl derivatives of DNJ having both glucosidase I inhibitory activity and antiviral activity, although no data on the long-chain N-alkyl derivatives was disclosed. See, e.g., DE 3,737,523, EP 315,017 and U.S. Patent Nos. 4,260,622; 4,639,436; and 5,051,407.

In another early study, although N-alkylation of the base DNJ reduced the concentration required for 50% inhibition of glucosidase I, the inhibitory activity was reduced as the length of the N-alkyl chain was increased from N-methyl to N-decyl according to Schweden et al., Arch. Biochem. Biophys. 248, 335-340, at 338 (1986).

As far as the antiviral activity of the amino-sugar compounds against any particular virus is concerned, the activity of any specific analog cannot be predicted in advance. For example, in biologic tests for inhibitory activity against the human immunodeficiency virus (HIV), slight changes in the structure of the N-substituent were shown to have pronounced effects upon the antiviral profile as reported by Fleet et al., FEBS Lett. 237, 128-132 (1988). As disclosed in U.S. Patent No. 4,849,430, the N-butyl derivative of DNJ was unexpectedly found to be more than two log orders more effective as an inhibitor of HIV than the N-methyl analog and three log orders more effective than the N-ethyl analog.

In another study of N-alkyl derivatives of DNJ for activity against glycolipid biosynthesis, the N-hexyl derivative of DNJ required a dose of 0.2 mg/ml, whereas the corresponding N-butyl analog required a dose of only 0.01-0.1. On the other hand, the N-methyl analog was inactive. Thus, it was believed that effective carbon chain length of the N-alkyl group for this activity ranged from 2 to 8 according to U.S. Patent No. 5,472,969. No disclosure was made therein concerning the N-nonyl or other long-chain N-alkyl derivatives of DNJ.

N-nonyl-DNJ has been reported to be effective as an inhibitor of the Hepatitis B virus (HBV) based on inhibition of alpha-glucosidases in the cellular endoplasmic reticulum (ER) according to Block et al., Nature Medicine 4(5) 610-614 (1998).

The effectiveness of the long-chain N-alkyl derivatives of DNJ in the method of the invention for treatment of Gaucher's disease and other such glycolipid storage diseases is illustratively demonstrated herein by inhibitory activity of N-nonyl and N-decyl DNJs against glycolipid biosynthesis in Chinese hamster ovary (CHO) cells and human myeloid (HL-60) cells.

CHO cells are known glycoprotein-secreting mammalian cells. A typical CHO cell line is CHO-K1 which is available to the public from the American Type Culture Collection, Bethesda, MD, under accession number ATCC CCL 61.

HL-60 cells are human promyelocytic cells described by Collins et al., Nature 270, 347-349 (1977). They are also readily available from the American Type Culture Collection under accession number ATCC CCL 240.

Effective activity of N-nonyl-DNJ also is further illustratively demonstrated herein in conventional bovine kidney cells (e.g., MDBK, ATCC CCL 22) and hepatoma cells (e.g., HepG2, ATCC HB 8065).

The unpredictability of the N-nonyl-DNJ against glycolipid biosynthesis is demonstrated herein by its inhibitory activity in the foregoing two cell lines. The N-nonyl-DNJ was unexpectedly found to be from about ten- to about twenty-fold better in the CHO cells and about four hundred times better in the HL-60 cells than N-butyl-DNJ at equivalent concentrations. The N-decyl-DNJ was demonstrated to be an effective inhibitor in HL-60 cells at 50 times lower concentrations than N-butyl-DNJ.

The N-nonyl-DNJ also exhibits a more dramatic difference than N-butyl-DNJ in uptake which permits its use at a substantially lower level. In tests of organ distribution, the N-nonyl-DNJ was taken up five times better into the brain than N-butyl-DNJ. Thus, the N-nonyl-DNJ is believed to be a substantially better compound than N-butyl-DNJ for treating glycolipid storage disorders which involve the non-systemic side.

N-nonyl-DNJ and N-decyl-DNJ can be conveniently prepared by the N-nonylation or N-decylation, respectively, of 1,5-dideoxy-1,5-imino-D-glucitol (DNJ) by methods analogous to the N-butylation of DNJ as described in Example 2 of U.S. Patent No. 4,639,436 by substituting an equivalent amount of n-nonylaldehyde or n-decylaldehyde for n-butylaldehyde. The starting materials are readily available from many commercial sources. For example, DNJ is available from Sigma, St. Louis, MO. n-Nonylaldehyde, also known as 1-nonanal or pelargonaldehyde, and n-decylaldehyde, also known as decanal, are commercially available from Aldrich, Milwaukee, WI. It will be appreciated, however, that the method of the invention is not limited to any particular method of

synthesis of the N-nonyl-DNJ, N-decyl-DNJ, or other long-chain N-alkyl derivatives of DNJ.

The N-nonyl-DNJ, N-decyl-DNJ, and other long-chain N-alkyl derivatives of DNJ, can be used for treatment of patients afflicted with Gaucher's disease and other glycolipid storage diseases by conventional methods of administering therapeutic drugs. Thus, the active compound is preferably formulated with pharmaceutically acceptable diluents and carriers. The active drug can be used in the free amine form or the salt form. Pharmaceutically acceptable salt forms are illustrated, e.g., by the HCl salt. The amount of the active drug to be administered must be an effective amount, that is, an amount which is medically beneficial against Gaucher's disease or other glycolipid storage disease but does not present adverse toxic effects which outweigh the advantages that accompany its use. It would be expected that the adult human daily dosage would normally range from about 0.1 to about 1000 milligrams of the active compound. The preferable route of administration is orally in the form of capsules, tablets, syrups, elixirs, gels and the like, although parenteral administration also can be used.

Suitable formulations of the active compound in pharmaceutically acceptable diluents and carriers in therapeutic dosage form can be prepared by the person skilled in the art by reference to general texts and treatises in the pharmaceutical field such as, for example, Remington's Pharmaceutical Sciences, Ed. Arthur Osol, 16 ed., 1980, Mack Publishing Co., Easton, PA, and 18th ed., 1990.

Other glycolipid storage diseases to which the method of the invention is directed are, e.g., Tay-Sachs disease, Sandhoff disease, Fabry disease, GM1 gangliosidosis and fucosidosis.

DETAILED DESCRIPTION OF THE INVENTION

While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the invention, it is believed that the invention will be better understood from the following preferred embodiments of the invention taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows thin layer chromatography of (a) CHO and (b) HL-60 treated cells. Cells were cultured for four days in the presence of radiolabelled palmitic acid and the following concentrations of compound:

- a) control, no compound
- b) 50 μ M NB-DNJ
- c) 5 μ M NB-DNJ
- d) 2.5 μ M NB-DNJ
- e) 0.25 μ M NB-DNJ
- f) 0.025 μ M NB-DNJ
- g) 50 μ M NN-DNJ
- h) 5 μ M NN-DNJ
- i) 2.5 μ M NN-DNJ
- j) 0.25 μ M NN-DNJ
- k) 0.025 μ M NN-DNJ

After extraction the radioactively labelled glycolipids were separated by TLC and visualized by radioautography.

FIG. 2, in two parts, FIG.2a and FIG.2b, shows double reciprocal plots of the inhibition of the ceramide glucosyltransferase by N-butyl-DNJ (NB-DNJ). HL-60 cell ceramide glucosyltransferase activity was measured using ceramide concentrations of 5-20 μ M (FIG.2a) and UDP-glucose concentrations of 0.59-5.9 μ M (FIG.2b). NB-DNJ concentrations of 5-100 μ M were used. The inhibition constants (K_i) were calculated by plotting

the Lineweaver-Burk slope against inhibitor concentration as shown in the inserts.

FIG. 3 shows inhibition of HL-60 cell ceramide glucosyltransferase activity by N-butyl-DNJ (open circles) and N-nonyl-DNJ (closed circles). Activity was expressed as a percentage of control without inhibitor and the IC_{50} values calculated from the rate curves shown. N-butyl-DNJ = $27.1 \mu M$; N-nonyl-DNJ = $2.8 \mu M$.

FIG. 4 shows structural relationship between NB-DNJ and ceramide glucosyltransferase substrate.

- (a) Ceramide structure from the crystal structure of glucosylceramide. The acceptor hydroxyl is on $C1^1$.
- (b) The structure NB-DNJ (N-alkyl) based on NMR studies and molecular modelling.
- (c) One possible overlay of ceramide and NB-DNJ.

FIG. 5, in two parts, FIG. 5A and FIG. 5B, shows bar graphs of estimated radioactivity. Radiolabelled N-butyl-DNJ (FIG. 5B) and N-nonyl-DNJ (FIG. 5A) were added to cultured CHO, MDBK and HepG2 cells for the times indicated. Cells were extensively washed and acid precipitated. After solution in NaOH, cell associated radioactivity was determined as a percentage of radiolabelled compound added.

FIG. 6 is a bar graph which shows organ distribution of radiolabelled N-butyl-DNJ (NB-DNJ) and N-nonyl-DNJ (NN-DNJ). Mouse body fluids and organs were collected for different times after gavage with radiolabelled compound. Radioactivity in each sample was determined and expressed as a percentage of radioactivity recovered. Solid bars, NN-DNJ, hatched bars, NB-DNJ.

FIG. 7 shows the structures of N-alkylated deoxynojirimycin exemplified herein. Note that the C16 and C18 N-alkyl chains contain an unsaturated bond at ten and twelve carbon atoms from the nitrogen, respectively, whereas the others are saturated.

FIG. 8 shows Inhibitory Constants of C4 to C18 DNJ Analogs for Ceramide Glucosyltransferase and α -Glucosidase. FIG.8 contains additional data to those seen in FIG.3 showing inhibition constants (IC_{50} , μM) for the N-alkyl series measured against ceramide glucosyltransferase (CerGlcT) and α -glucosidase. The trend is similar to the FIG.3 description - increasing chain length increases inhibition for glucosyltransferase, but not for glucosidase.

FIG. 9 shows C4 to C18 DNJ Analog Uptake in MBDK Cells in which radioactivity incorporation/cpm protein is plotted against time in hours (h). FIG.9 shows additional data to those shown in FIG.5 using C4-C18 N-alkylated DNJ compounds. Trend is apparent - increasing chain length increases cellular uptake in a time-dependent fashion. The double bond has some effect here since the unsaturated C16 and C18 analogs show similar kinetics to the fully saturated C10 and C12 analogs, respectively.

FIG. 10 shows Distribution of N-Alkylated DNJ Analogs in Mouse Liver. The radioactivity recovered (%) is plotted against N-alkyl chain length (C4 to C18) for 30 minutes (clear bars), 60 minutes (shaded bars) and 90 minutes (filled, black bars). FIG.10 shows the results of oral gavage with radiolabelled N-alkylated compounds using methods described in FIG.6. Short chain compounds (C4-C6) are rapidly cleared in a time-dependent manner. The C9 and C10 compounds show increased deposition and slower clearance. The C12 to C18 analogs show the reverse trend, i.e., reduced appearance in the liver but this increases with time.

FIG. 11 shows Distribution of N-alkylated DNJ Analogs in Mouse Brain. The radioactivity recovered (%) is plotted against N-alkyl chain length (C4 to C18) for 30 minutes (clear bars), 60 minutes (shaded bars) and 90 minutes (filled, black bars). FIG.11 shows that the progressive accumulation that is also seen in the brain has slowed kinetics suggesting that there is reduced adsorption of longer alkyl chain compounds from the gut.

FIG. 12 is a series of four bar charts, A, B, C and D, in which radioactivity (cpm) found in the liver is plotted against time post gavage in hours (h) with four different N-alkyl analogs of deoxynojirimycin (DNJ). The four analogs shown are: FIG.12A, N-butyl(C4); FIG.12B, N-nonyl (C9); FIG.12C, N-dodecyl(C12); FIG.12D, N-cis-13-octadecenyl (C18). FIG.12 shows that in the liver the majority of radioactive C4 is found after 1.5 h but with increasing chain length the clearance time is gradually increased with C18 showing significant deposition at 24 h post gavage.

FIG. 13 is a series of four bar charts, A, B, C and D, in which radioactivity (cpm) found in the brain is plotted against time post gavage in hours (h) with the same analog compounds as in FIG.12. The four analogs shown are: FIG.13A, N-butyl (C4); FIG.13B, N-nonyl (C9); FIG.13C, N-dodecyl (C12); FIG.13D, N-cis-13-octadecenyl (C18). FIG.13 shows that the same effect as in the liver in FIG.12 is seen in the brain but at much longer time points, reflecting reduced transmission from the gut to blood and hence, brain.

FIG. 14 shows Imino Sugar (N-alkyl DNJ) Binding to Serum Protein. The percentage compound radioactivity is plotted against N-alkyl chain length (C4 to C18) with the protein bound percentage shown by open circles and the non-bound percentage shown by filled circles. FIG.14 shows the protein binding capacity of N-alkylated compounds. Short chain compounds (C4-C6) bind poorly but those larger than C10 are almost completely bound

to protein. The C8 and C9 analogs appear to favor equally, protein and solution phase.

In order to illustrate the invention in greater detail, the following specific laboratory examples were carried out. Although specific examples are thus illustrated herein, it will be appreciated that the invention is not limited to these specific, illustrative examples or the details therein.

EXAMPLE I.

A comparison was made between N-butyl-DNJ and N-nonyl-DNJ for glycolipid biosynthesis inhibition which showed that potency is cell and chain length dependent. Chinese Hamster Ovary (CHO) cells and human myeloid (HL-60) cells grown in the presence of varying concentrations of inhibitor in addition to a precursor (radiolabelled palmitic acid) of glycolipid biosynthesis were treated with solvents to extract the glycolipids by the procedure described by Platt et al., J. Biol. Chem. 269, 8362-8365 (1994).

The radiolabelled lipids were separated by TLC (FIG.1) and bands corresponding to glucosylceramide and lactosylceramide were quantitated by scanning densitometry to estimate the reduction in glycolipid biosynthesis. These data were plotted to obtain inhibitory constants (IC_{50}) for both cell lines and compounds (Table 1).

These data show that cell lines have different sensitivities to both N-butyl- and N-nonyl-DNJ. HL-60 cells are more than 10 times more sensitive to N-butyl-DNJ and 100 times more sensitive to N-nonyl-DNJ than CHO cells. This cell specificity is unexpected. In addition, N-nonyl is between 10 and 365 times more effective than N-butyl-DNJ.

Detailed work to probe the mechanism of the ceramide glucosyltransferase, the enzyme inhibited by alkylated deoxynojirimycin compounds has demonstrated that these compounds are competitive inhibitors for ceramide and non-competitive inhibitors for UDP-glucose (FIG.2). N-nonyl-DNJ has a 10-fold increased potency over N-butyl-DNJ in inhibiting ceramide glucosyltransferase in *in vitro* assays (IC_{50} values of 2.8 μ M and 27.1 μ M respectively, see FIG.3).

The mechanism of action of alkylated deoxynojirimycin compounds is proposed to be that of ceramide mimicry and a model demonstrating this mimicry at the molecular level is shown in FIG.4. An energy minimized molecular model of NB-DNJ and ceramide predicts structural homology of three chiral centers and the N-alkyl chain of NB-DNJ, with the *trans*-alkenyl and N-acyl chain of ceramide. This increased *in vitro* potency does not explain the dramatic difference in inhibition of glycolipid biosynthesis in cellular systems.

The activity is explained by the differential uptake into cells. In three cell lines, CHO, MDBK and HepG2, radio-labelled N-nonyl-DNJ and N-butyl-DNJ were incubated for up to 24 hours and the amount of cell-associated radioactivity determined. In all cases N-nonyl-DNJ was increased by 3.5-5 fold. It is clearly the combination of the inhibitory effect and increased uptake that is important in potentiating the inhibition by N-nonyl-DNJ.

Further evidence that longer alkyl chains are taken up much better has been obtained by *in vivo* studies with mouse. After oral gavage with radiolabelled N-nonyl-DNJ and N-butyl-DNJ for 30, 60, and 90 minutes, the body fluids were collected and organs removed for estimations of radio activity (FIG.5). The amount of radioactivity recovered in the liver and brain was 10 fold higher for N-nonyl-DNJ than N-butyl-DNJ after 90 min (see Table 2).

Evidence was obtained that longer (than C9) chain DNJ compounds are more effect ceramide glucosyltransferase inhibitors. This follows from proposed mechanism of action studies that demonstrate enhanced potency correlates with ceramide mimicry (FIG.4). More specifically, N-decyl-DNJ (C10) shows inhibition at 50 times lower concentrations than N-butyl-DNJ in the HL-60 cell-based assay described above. In view of the above data, the long-chain N-alkyl derivatives of DNJ are effective for treatment of glycolipid storage diseases.

TABLE 1

Cells	N-butyl-DNJ (IC_{50} , μM)	N-nonyl-DNJ (IC_{50} , μM)
CHO	25-50	2-2.7
HL-60	1.8-7.3	0.02-0.4

Table 1. Inhibition of glycolipids of N-butyl- and N-nonyl-DNJ. Radiolabelled glucosylceramide and lactosylceramide bands from Fig. 1 were quantitated by scanning densitometry and the percentage of control (no treatment, track a, Fig. 1) expressed in comparison to compound dose. From the linear curve, an IC_{50} value was obtained. A range of values is quoted to represent variability of the radiolabelled products.

TABLE 2

<u>Time (min)</u>	<u>% recovered N-nonyl-DNJ</u>		<u>% recovered N-butyl-DNJ</u>	
30	Liver	Brain	Liver	Brain
60	27.1	0.4	8.5	0.2
90	12.6	0.3	2.8	0.1
	13.5	0.4	0.9	0.03

Table 2. Recovery of radiolabelled compounds after administration in the normal mouse. Mouse body fluids and organs were collected for different times after gavage with

radiolabelled compound. Radioactivity in each sample was determined and expressed as a percentage of radioactivity recovered (data from Fig.5).

EXAMPLE II

The laboratory procedures of Example I were carried out to further demonstrate the advantage of the long-chain N-alkyl derivatives of deoxynojirimycin compared to the short-chain analogs for the treatment of glycolipid storage diseases. The chemical structures of the analogs compared in this Example are shown in Figure 7. These analogs are saturated except the C16 and C18 alkyl chain analogs which are mono-unsaturated.

The inhibition constants (IC_{50}) for the N-alkyl series measured against ceramide glycosyltransferase (CerGlcT) and alpha-glucosidase are shown in Figure 8. The trend is similar to that shown in Figure 3 in which increasing chain length increases inhibition for glycosyltransferase, but not for glucosidase. This supports the mechanism of ceramide mimicry as the basis of inhibition shown in Figure 4. The optimal chains length appears to be C10 (decyl).

Figure 9 confirms the trend shown in Figure 5 in which increasing chain length increases cellular uptake in a time dependent manner. The effect of the double bond in the C16 and C18 analogs is seen in that the C16 shows similar kinetics to the saturated C10 analog, and the C18 shows similar kinetics to the saturated C12 analog.

In Figure 10, the results of oral gavage with radiolabelled analogs as in Figure 6 are shown for additional analogs. Short-chain analogs (C4 to C6) are cleared rapidly in a time dependent manner. The C9 and C10 analogs show increased deposition and slower clearance. The C12 to C18 analogs show reduced appearance

in the liver, but this increases with time. These results support the mechanism of increased tissue uptake by longer alkyl chain analogs since after 30 minutes the accumulation in the liver of the C9 analog is ten times that seen with the short-chain C4 analog.

Figure 11 shows the progressive accumulation that is also seen in the mouse brain has slowed kinetics and thereby suggests that there is a reduced adsorption of the longer chain alkyl analogs from the gut.

Further evidence of reduced adsorption is shown in Figures 12 and 13 when longer time points post gavage are used to monitor tissue deposition. Thus, Figure 12 shows that in the liver the majority of radioactive C4 is found after 1.5 hours, but with increasing chain length the clearance time is gradually increased, with C18 showing significant deposition at 24 hours post gavage. Figure 13 shows that the same effect is seen in the mouse brain but at much longer time points, reflecting reduced transmission from the gut to the blood and hence the brain.

Figure 14 shows the protein binding capacity of the N-alkylated analogs of deoxynojirimycin. The short-chain analogs (C4 to C6) bind poorly but those larger than C10 are almost completely bound to protein. The C8 and C9 analogs appear to favor equally, protein and solution phase.

In summary then, the slowed uptake from the gut by the long-chain alkyl analogs of deoxynojirimycin shown in Example II results in slowed transmission to the liver but there is progressive accumulation. This accumulation in the liver with time is also shown in the brain. These results have great significance for the treatment of glycolipid storage diseases, especially when the storage in the brain shows pathology for Gaucher type II/III, Tay-Sachs and Sandhoff diseases.

Various other examples will be apparent to the person skilled in the art after reading the present disclosure without departing from the spirit and scope of the invention. It is intended that all such other examples be included within the scope of the appended claims.

CLAIMS

What is claimed is:

1. The method of treating a patient affected with a glycolipid storage disease comprising administering to said patient a long-chain N-alkyl derivative of deoxynojirimycin having from nine to about twenty carbon atoms in the alkyl chain in an amount effective for alleviating or inhibiting said glycolipid storage disease.
2. The method of Claim 1 in which the long-chain N-alkyl derivative of deoxynojirimycin is N-nonyl-DNJ or N-decyl-DNJ.
3. The method of Claim 2 in which the N-alkyl derivative of deoxynojirimycin is N-nonyl-DNJ.
4. The method of Claim 1 in which the glycolipid storage disease is Gaucher's disease.
5. The method of Claim 2 in which the glycolipid storage disease is Gaucher's disease.
6. The method of Claim 3 in which the glycolipid storage disease is Gaucher's disease.
7. The method of Claim 1 in which the N-alkyl derivative of deoxynojirimycin is administered in a dosage of from about 0.1 to about 1000 mg in a pharmaceutically acceptable diluent or carrier.
8. The method of Claim 2 in which the N-nonyl-DNJ or N-decyl-DNJ is administered in a dosage of from about 0.1 to about 1000 mg in a pharmaceutically acceptable diluent or carrier.

9. The method of Claim 3 in which the N-nonyl-DNJ is administered in a dosage of from about 0.1 to about 1000 mg in a pharmaceutically acceptable diluent or carrier.

10. The method of Claim 4 in which the N-alkyl derivative of deoxynojirimycin is administered in a dosage of from about 0.1 to about 1000 mg in a pharmaceutically acceptable diluent or carrier.

11. The method of Claim 5 in which the N-nonyl-DNJ or N-decyl-DNJ is administered in a dosage of from about 0.1 to about 1000 mg in a pharmaceutically acceptable diluent or carrier.

12. The method of Claim 6 in which the N-nonyl-DNJ is administered in a dosage of from about 0.1 to about 1000 mg in a pharmaceutically acceptable diluent or carrier.

FIG. 1B

HL60 cells

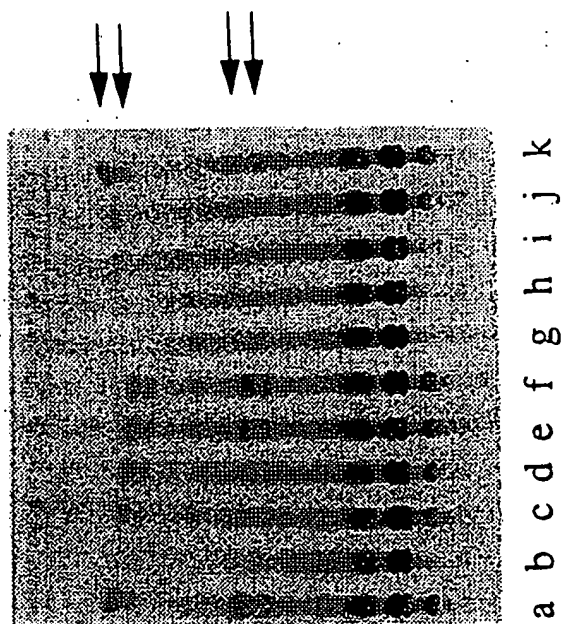
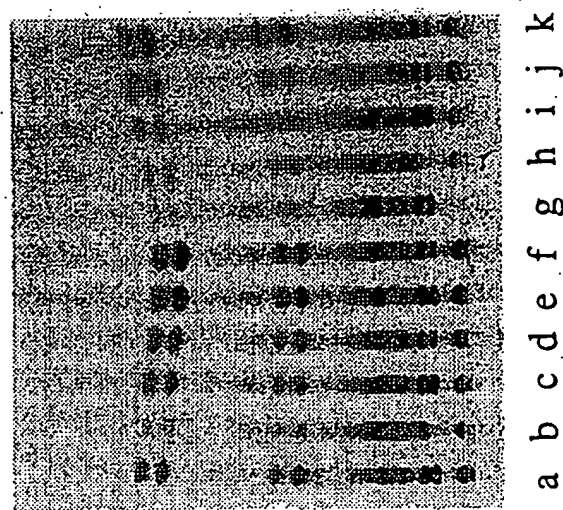


FIG. 1A

CHO cells



GlcCer
LacCer
origin

2/14

FIG.2A

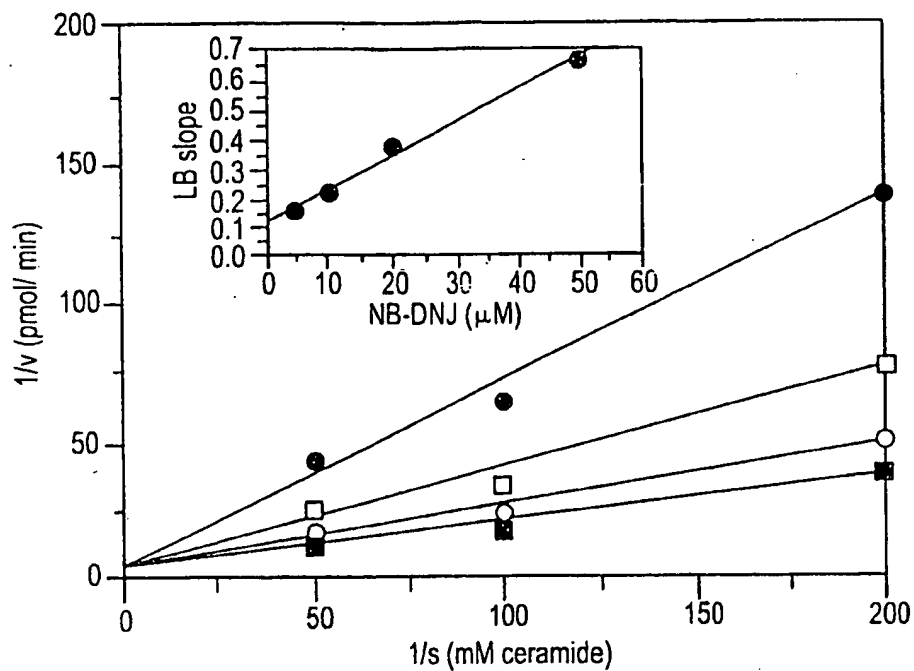


FIG.2B

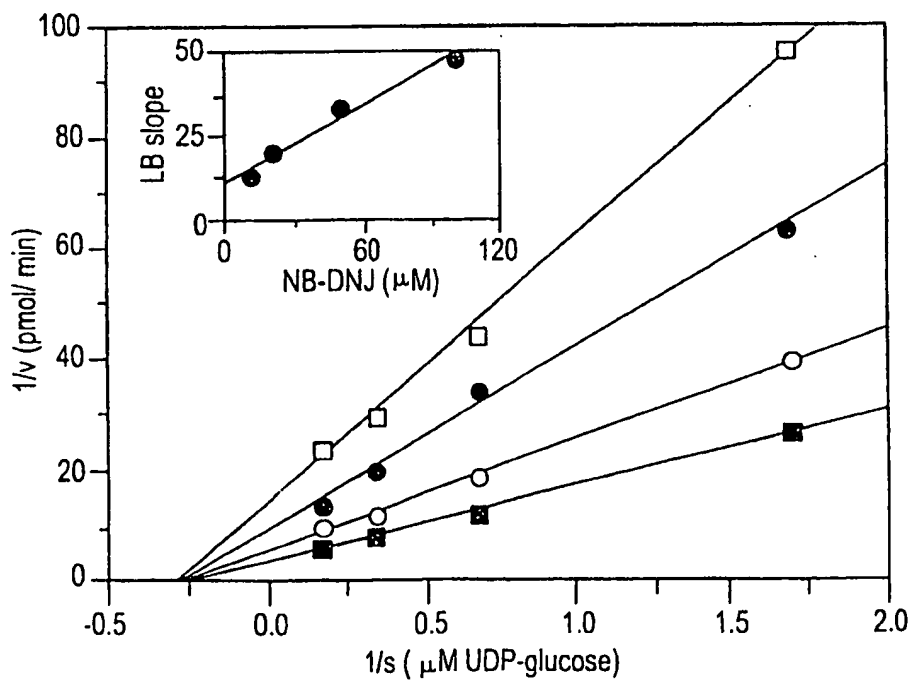


FIG.3

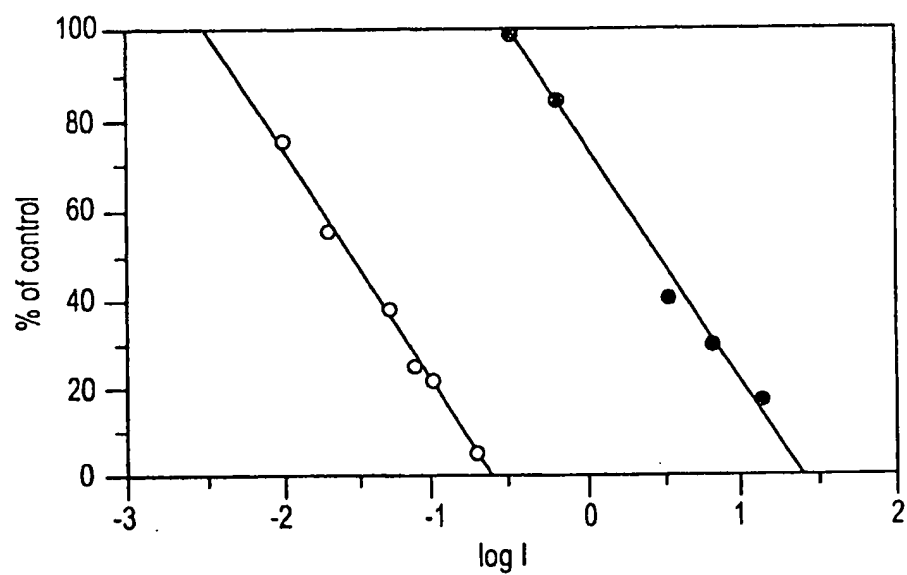


FIG.4C

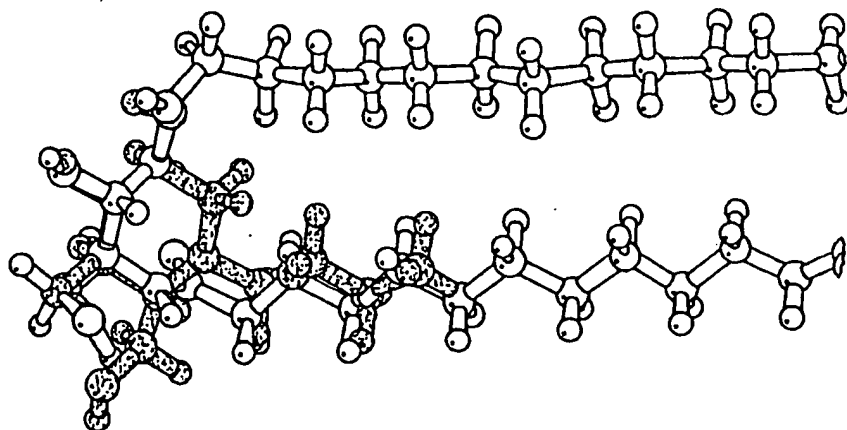


FIG.4B

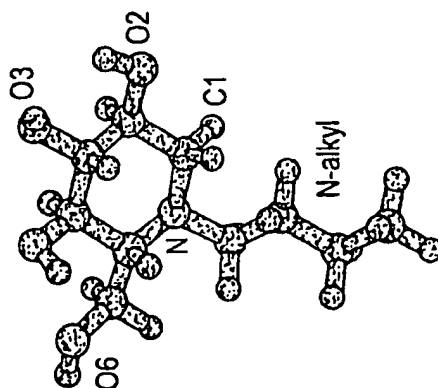


FIG.4A

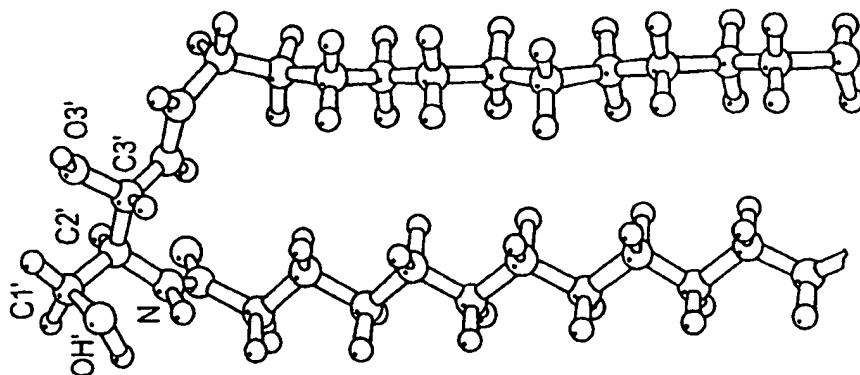


FIG.5A

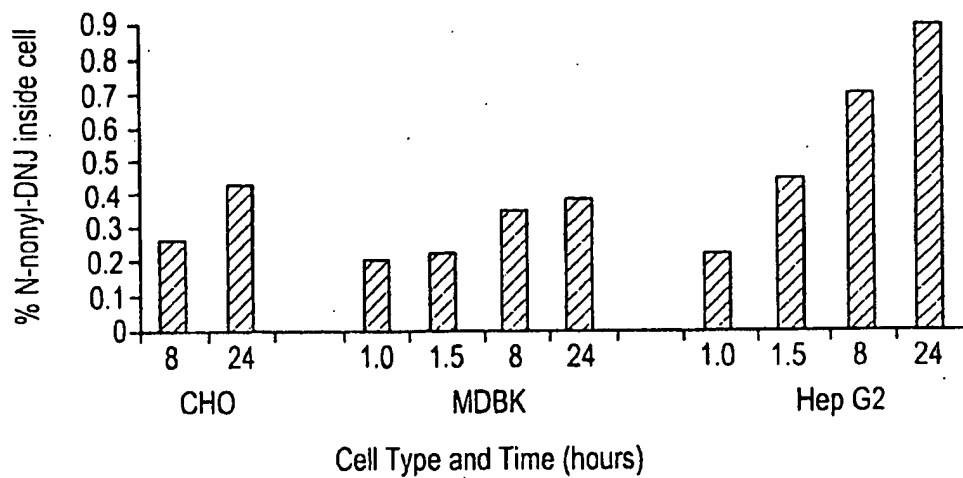


FIG.5B

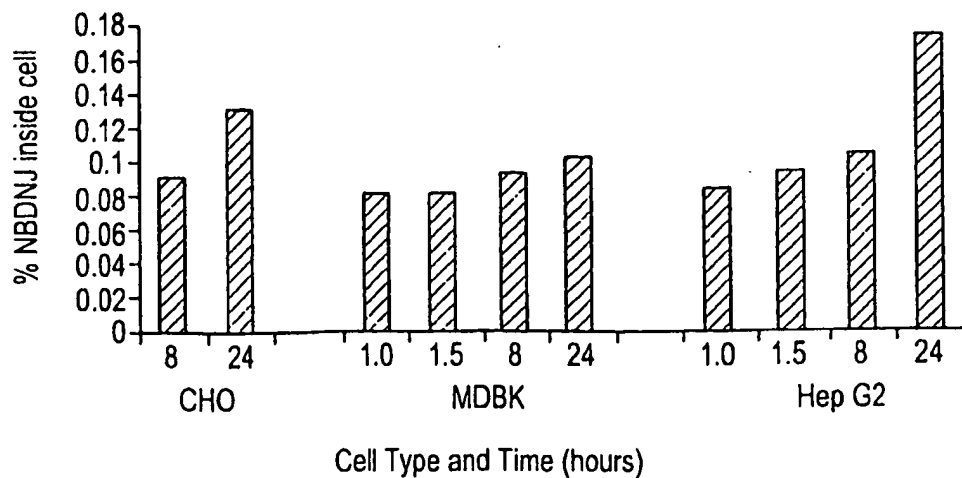


FIG.6

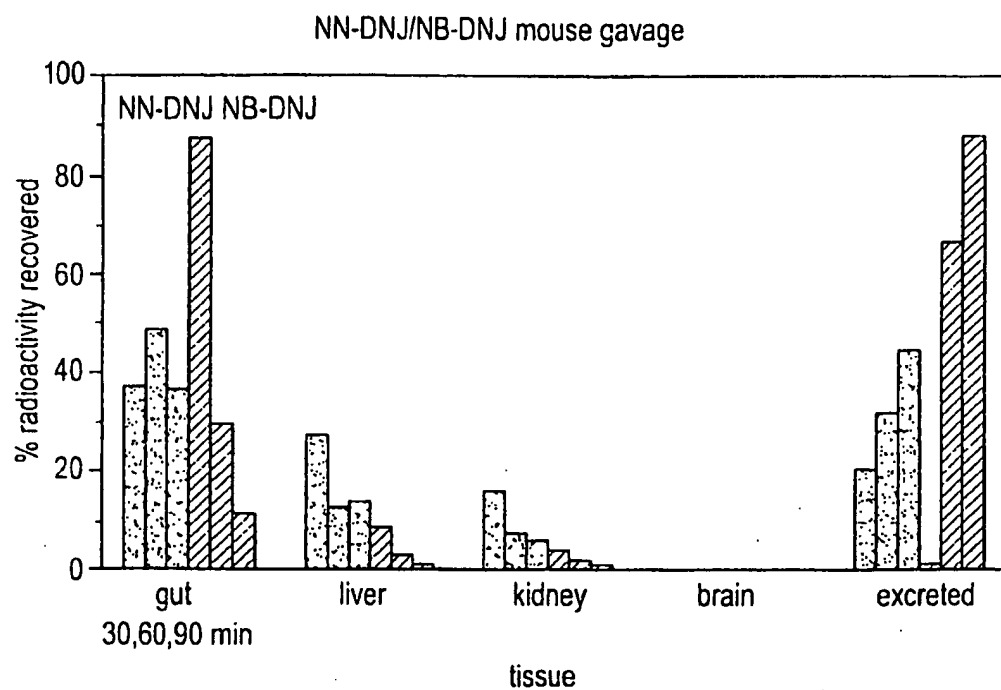
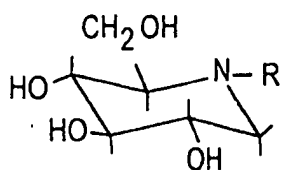


FIG.7

N-alkylated deoxynojirimycin

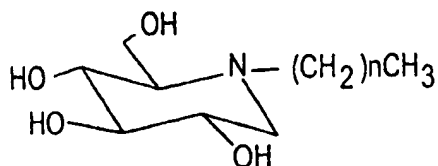


R=

- CH₂CH₂CH₂CH₃
- CH₂CH₂CH₂CH₂CH₃
- CH₂CH₂CH₂CH₂CH₂CH₃
- (CH₂)₆CH₃
- (CH₂)₆CH₂CH₃
- (CH₂)₆CH₂CH₂CH₃
- (CH₂)₆CH₂CH₂CH₂CH₃
- (CH₂)₆CH₂CH₂CH₂CH₂CH₂CH₃
- CH₂(CH₂)₉CHCH(CH₂)₃CH₃
- CH₂(CH₂)₁₁CHCH(CH₂)₃CH₃

FIG.8

Inhibitory Constants of C4-C18 DNJ Analogues for Ceramide
Glucosyltransferase and α -Glucosidase



Chain length	CerGlcT (IC ₅₀ , μ M)	α -Glucosidase (IC ₅₀ , μ M)
4	34.4	0.57
5	26.8	
6	23.8	
8	16.8	
9	7.4	
10	3.1	0.48
12	5.2	
16	3.4	
18	4.1	

FIG.9

C4-C18 DNJ Analogue Uptake in MDBK Cells

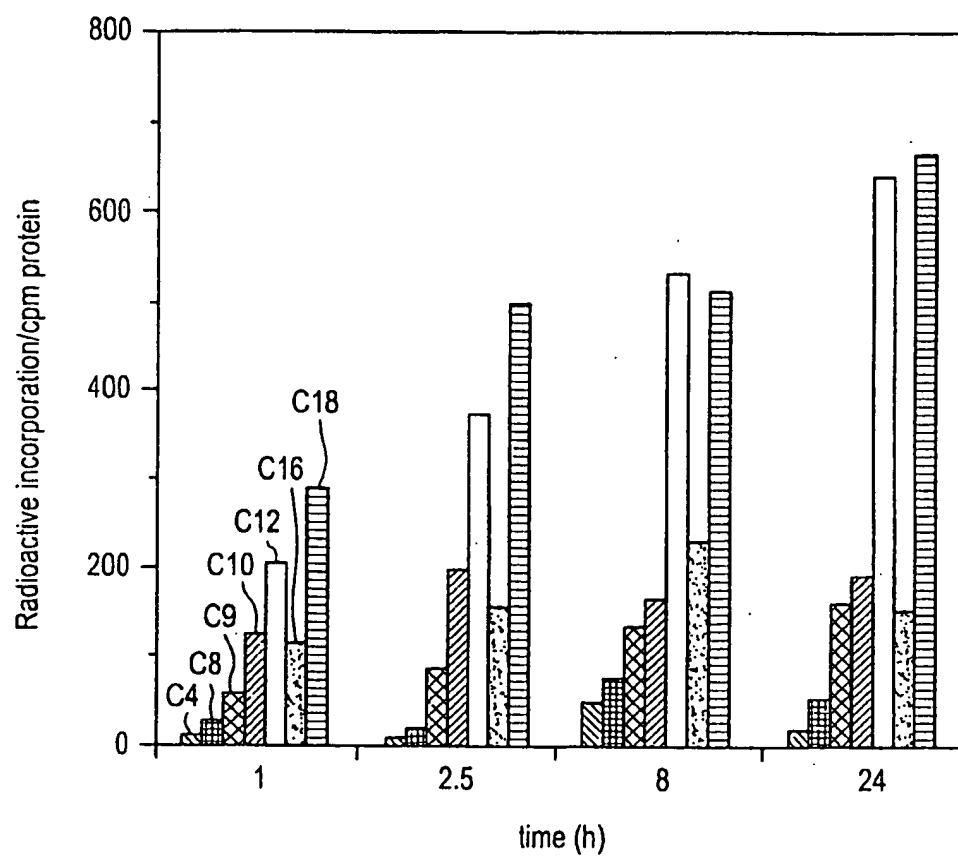


FIG.10

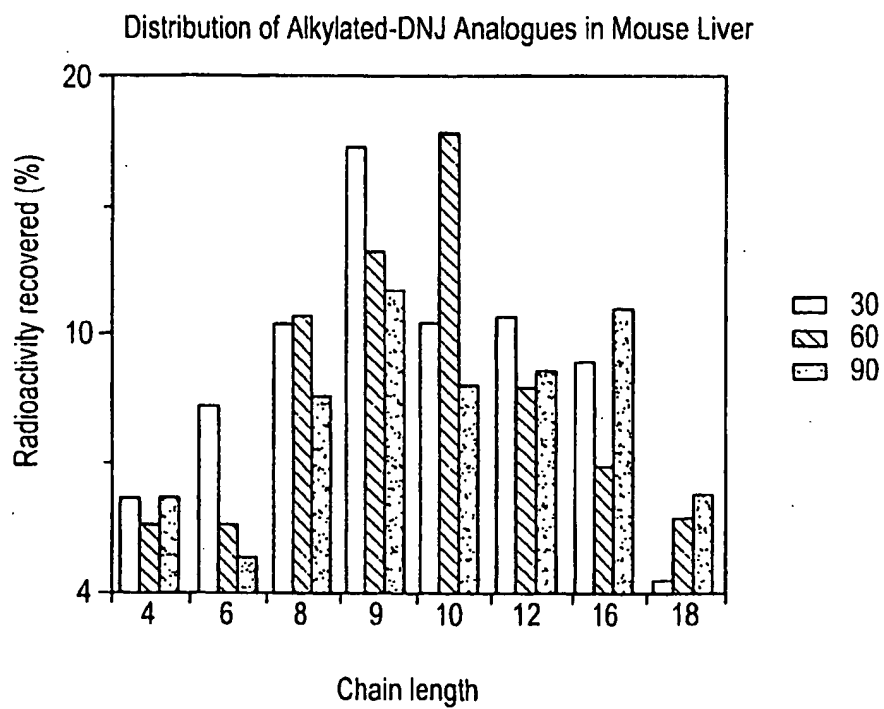


FIG.11

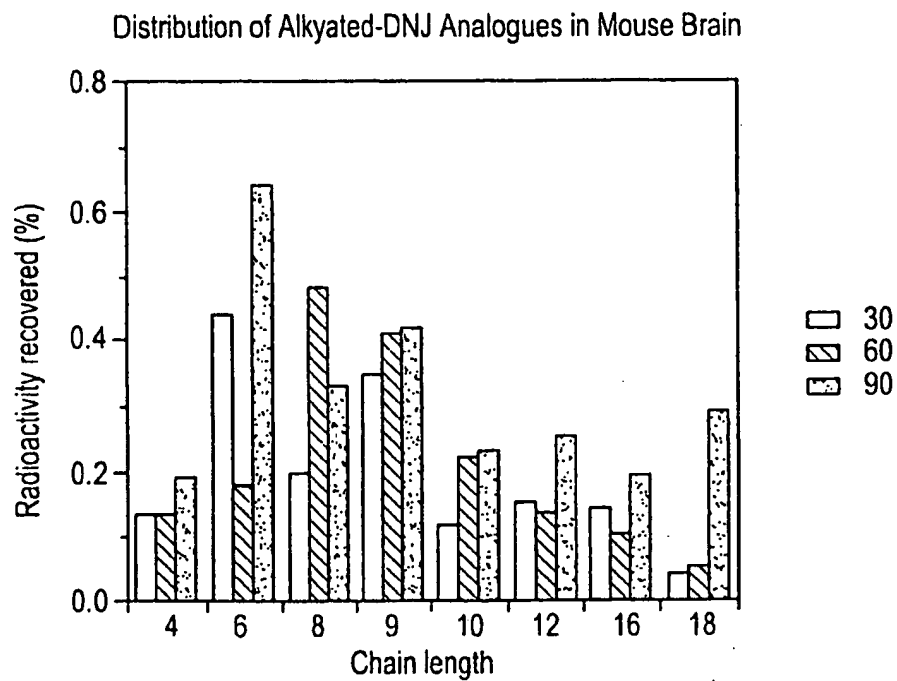


FIG.12A

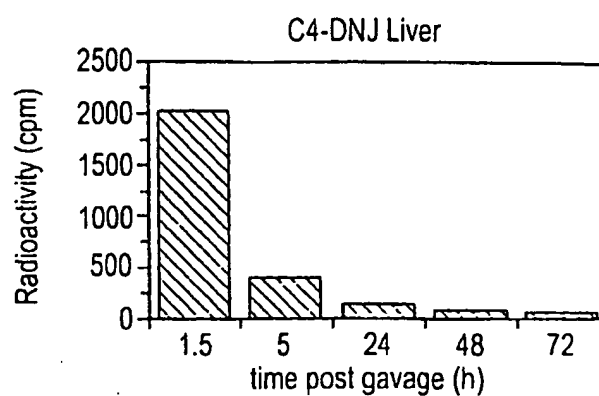


FIG.12B

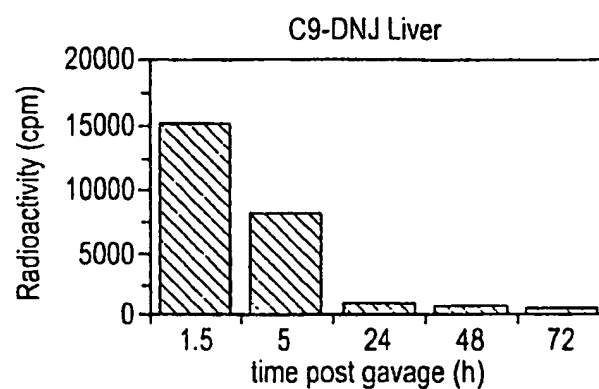


FIG.12C

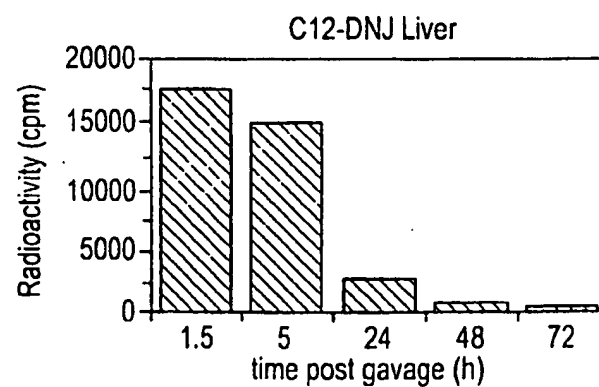


FIG.12D

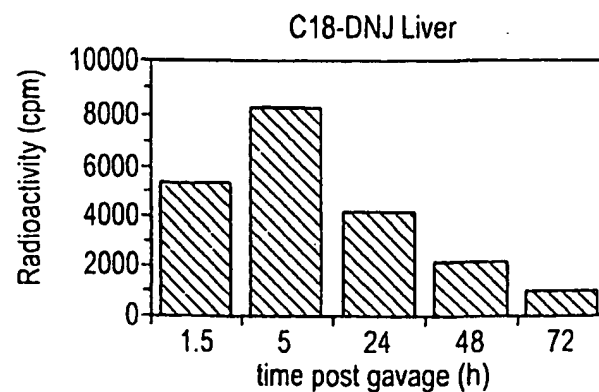


FIG.13A

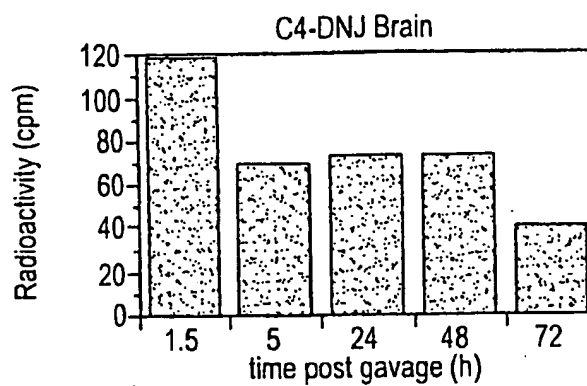


FIG.13B

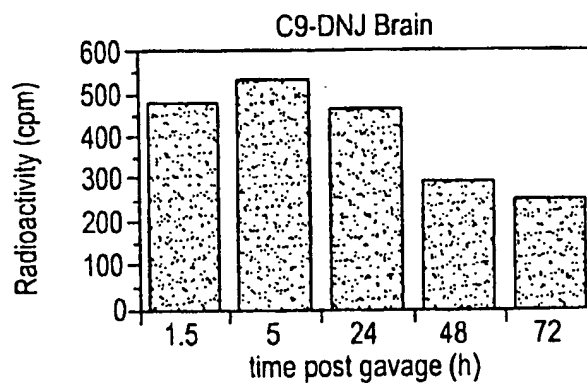


FIG.13C

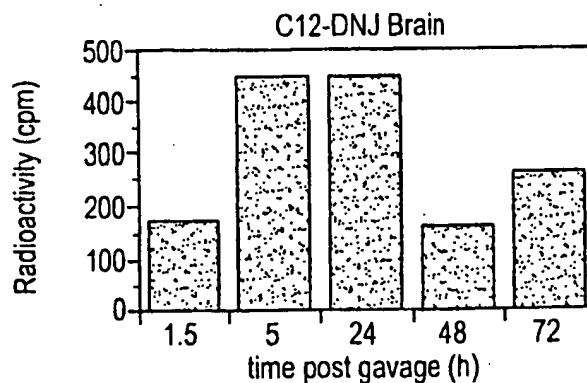


FIG.13D

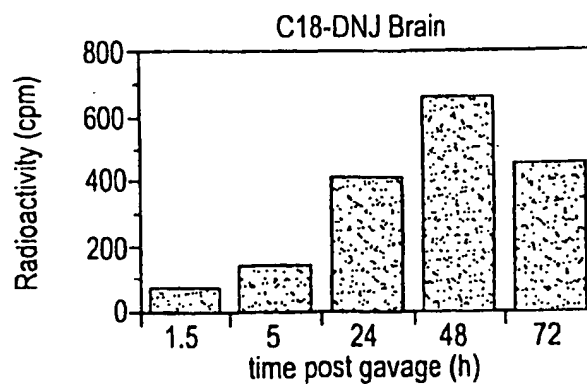
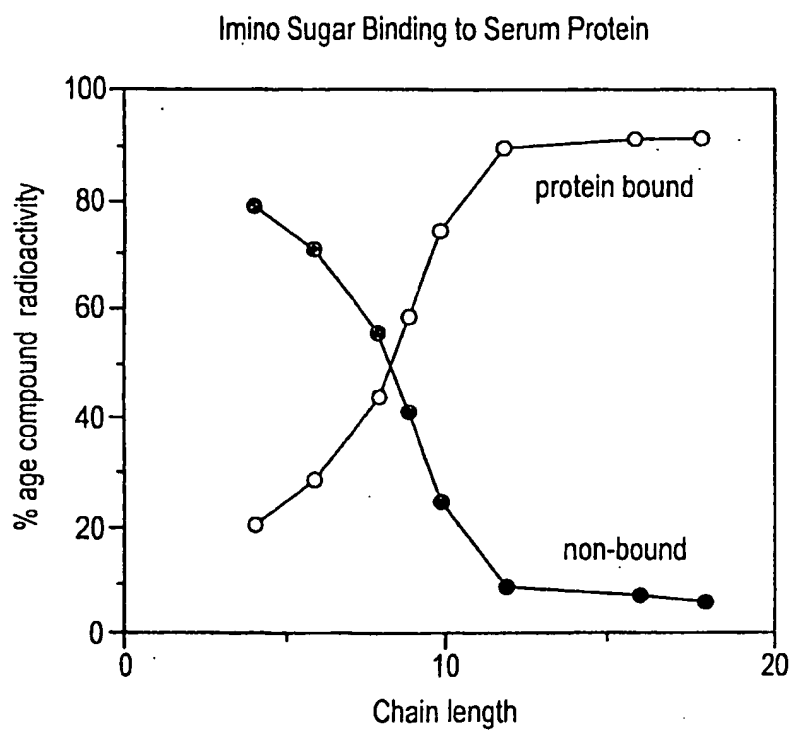


FIG.14



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/27918

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K31/445

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 98 02161 A (UNIVERSITEIT VAN AMSTERDAM) 22 January 1998 (1998-01-22) the whole document	1-12
A	EP 0 193 770 A (BAYER AG.) 10 September 1986 (1986-09-10) page 1, line 15 -page 2, line 27 -/-	1-12



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Economou, D

INTERNATIONAL SEARCH REPORT

Int'l. Application No.
PCT/US 99/27918

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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A	<p>PLATT ET AL.: "N-BUTYLDEOXYNOJIRIMYCIN IS A NOVEL INHIBITOR OF GLYCOLIID BIOSYNTHESIS" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 11, 18 March 1994 (1994-03-18), pages 8362-8365, XP000615445 USA cited in the application abstract page 8363, left-hand column, paragraph 4 -page 8365, left-hand column, paragraph 4</p>	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. l. Application No

PCT/US 99/27918

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9802161 A	22-01-1998	AU 3464797 A EP 0912179 A	09-02-1998 06-05-1999
EP 0193770 A	10-09-1986	DE 3507019 A JP 61200967 A	28-08-1986 05-09-1986



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K 31/445, A61P 35/00	A1	(11) International Publication Number: WO 00/56334 (43) International Publication Date: 28 September 2000 (28.09.00)
(21) International Application Number: PCT/US00/06933 (22) International Filing Date: 17 March 2000 (17.03.00) (30) Priority Data: 60/125,169 19 March 1999 (19.03.99) US 60/135,351 21 May 1999 (21.05.99) US 60/148,215 12 August 1999 (12.08.99) US (71) Applicants (for all designated States except US): THE TRUSTEES OF BOSTON COLLEGE [GB/GB]; Office of Research Administration, Boston College, McGuinn Hall 600, 1400 Commonwealth Avenue, Chestnut Hill, MA 02467-3809 (US). THE CHANCELLOR, MASTERS AND SCHOLARS OF THE UNIVERSITY OF OXFORD [GB/GB]; University Offices, Wellington Square, Oxford OX1 2JD (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): SEYFRIED, Thomas, N. [US/US]; 15 Shoreline Drive, Foxboro, MA 02035 (US). PLATT, Frances [GB/GB]; 33 Millwood End, Long Hanborough, Oxfordshire OX8 8BN (GB). RANES, Michaela, K. [US/US]; Apartment #1, 116 Sutherland Road, Brighton, MA 02135 (US).		(74) Agents: KOKULIS, Paul, N. et al.; Pillsbury Madison & Sutro, LLP, 1100 New York Avenue, NW, Washington, DC 20005 (US). (81) Designated States: JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.
(54) Title: USE OF IMINO SUGARS FOR ANTI-TUMOR THERAPY (57) Abstract The invention relates to methods for inhibiting the growth of tumors or other neoplasms, treating the symptoms that might be a consequence of such tumors or other neoplasms. It relates particularly to the formulation and/or administration of an effective amount of at least one of imino sugars or pharmaceutical acceptable salts thereof, 1-deoxynojirimycin (DNJ) or derivatives thereof, and glycosidase inhibitors or pharmaceutically acceptable salts thereof useful in such methods.		

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USE OF IMINO SUGARS FOR ANTI-TUMOR THERAPY

FIELD OF THE INVENTION

The invention relates to methods for inhibiting the growth of tumors or other neoplasms, or treating the symptoms that might be a consequence of such tumors or other neoplasms. It relates particularly to the formulation and/or administration of an effective amount of a pharmaceutical compound according to the invention.

BACKGROUND OF THE INVENTION

Antitumor therapy now involves an attack on the development of malignant tumor tissue by disrupting normal metabolic processes on which the new tumor depends for growth.

Tumor growth, like the growth of normal tissues, requires the synthesis of certain cell surface glycoproteins and glycolipids. Intracellular oligosaccharide processing depends on glycosidases and glycosyl transferases that can modify the structure and composition of these glycoproteins and glycolipids. It has been known for some time that glucosidase and glycolipid synthesis inhibitors, *e.g.*, the glucose analog N-butyl-1,5-deoxy-1,5-imino-D-glucitol (N-butyl DNJ), alter the synthesis of complex oligosaccharides. By altering these structures in endothelial cells with a related inhibitor castanospermine, it was possible to inhibit tumor growth (Pili *et al.*, *Cancer Res* 33:2920-2925, 1995; Radin, *Biochem Pharmacol* 15:589-595, 1999). Use of N-butyl-1,5-deoxy-1,5-imino-D-glucitol (N-butyl DNJ) to inhibit *in vitro* and *in vivo* growth of EPEN and CT-2A brain tumors was published after the filing date of our priority documents (Ranes *et al.*, *Proc Am Soc Cancer Res* 41:258, 2000).

Many of the existing drugs, however, are poorly tolerated by individuals such that the ratio of minimum dose with therapeutic effect to maximum dose that can be safely given is low. Moreover, it can be difficult to achieve a therapeutic concentration of these drugs in some regions of the body (*e.g.*, brain cancers). There is a need for more effective drugs to treat tumors and other neoplasia, especially to inhibit the growth thereof.

Other advantages of the invention are discussed below or would be apparent to a person skilled in the art of cancer prevention and treatment from that discussion.

SUMMARY OF THE INVENTION

An objective of the invention is to provide to provide imino sugars and derivatives thereof that are effective in the treatment of tumors and other neoplastic growths.

One embodiment of the invention is treatment of a tumor or other neoplasm with an imino sugar or pharmaceutically acceptable salt thereof. The amount of imino sugar or pharmaceutically acceptable salt thereof that is administered to an individual in need of such treatment is effective to slow and/or reduce growth of the tumor or other neoplasm in comparison to not treating the disease.

Another embodiment of the invention is treatment of a tumor or other neoplasm with 1-deoxynojirimycin (DNJ) or a derivative thereof. The amount of DNJ or derivative thereof that is administered to an individual in need of such treatment is effective to slow and/or reduce growth of the tumor or other neoplasm in comparison to not treating the disease.

A further embodiment of the invention is treatment of a tumor or other neoplastic growth (*i.e.*, a neoplasm) with a glycosidase inhibitor or pharmaceutically acceptable salt thereof. The amount of glycosidase inhibitor or pharmaceutically acceptable salt thereof that is administered to an individual in need of such treatment is effective to slow and/or reduce growth of the tumor or other neoplasm in comparison to not treating the disease.

Compounds of the invention may be used to produce a medicament or other pharmaceutical composition to treat a tumor or other neoplasm.

In particular, a long chain N-alkyl derivative of DNJ (*e.g.*, between five and 16 carbons, inclusive, in length) is preferred for use in the invention. More preferred are long chain N-alkyl derivatives between eight and 16 carbons, inclusive, in length. N-nonyl-1,5-deoxy-1,5-imino-D-glucitol (N-nonyl DNJ) is a preferred derivative. Short chain N-alkyl derivatives of DNJ (*i.e.*, four carbons or less in length) may be used, but are not preferred.

Treatment with a compound that has superior bioavailability and does not lower blood glucose levels is preferred, but not necessary, to achieve the objective of the invention.

BRIEF DESCRIPTION OF THE DRAWING

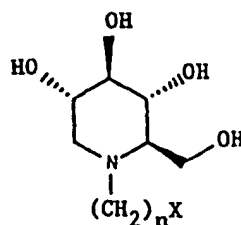
Figure 1 shows the inhibiting effect of N-alkyl imino sugars N-butyl DNJ and N-nonyl DNJ on experimental brain tumor growth in mice. Squares are control, diamonds are N-nonyl DNJ at 50 mg/kg/day, circles are N-nonyl DNJ at 500 mg/kg/day, and triangles are N-butyl DNJ at 500 mg/kg/day (*n.b.*, diamond and circle symbols are close to each other).

DETAILED DESCRIPTION OF THE INVENTION

In general, a tumor or other neoplasm may be treated with an imino sugar or pharmaceutically acceptable salt thereof. The amount of imino sugar or pharmaceutically acceptable salt thereof that is administered to an individual in need of such treatment is effective to slow and/or reduce growth of the tumor or other neoplasm in comparison to not treating the cancer. The imino sugar may be a galactoside analog. Preferably, the imino sugar has an N-alkyl chain of at least five carbons and, more preferably, the imino sugar contains at least one C₅-C₁₆ substituent. Bioavailability of the imino sugar across said individual's blood-brain barrier is substantially better than N-butyl DNJ. For example, the imino sugar may be N-nonyl DNJ.

The tumor or other neoplasm may be treated with 1-deoxynojirimycin (DNJ) or a derivative thereof. The amount of DNJ or derivative thereof that is administered to an individual in need of such treatment is effective to slow and/or reduce growth of the tumor or other neoplasm in comparison to not treating the cancer. Preferably, the derivative has an N-alkyl chain of at least five carbons and, more preferably, the derivative contains at least one C₅-C₁₆ substituent. Bioavailability of the derivative across said individual's blood-brain barrier is substantially better than N-butyl DNJ. For example, the derivative may be N-nonyl DNJ.

The tumor or other neoplasm may also be treated with an effective amount of a glycosidase inhibitor or pharmaceutically acceptable salt thereof. The amount of glycosidase inhibitor or pharmaceutically acceptable salt thereof that is administered to an individual in need of such treatment is effective to slow and/or reduce growth of the tumor or other neoplasm in comparison to not treating the cancer. The glycosidase inhibitor may be 1-deoxynojirimycin (DNJ) or a derivative thereof according to Formula A,



A

wherein X may be an unsaturated straight aliphatic hydrocarbon, saturated and unsaturated branched aliphatic hydrocarbon, aromatic hydrocarbon or substituted derivatives thereof, cyclic hydrocarbon or substituted derivatives thereof, -O-Y, -S-Y, -Y-OH, -Y-NH₂, -Y-COOH; -Y-CON⁺-R, or -Y-COO-R; wherein Y may be a saturated or unsaturated hydro-

carbon, that can be a straight aliphatic hydrocarbon, branched aliphatic hydrocarbon, aromatic hydrocarbon or substituted derivatives thereof, or cyclic hydrocarbon or substituted derivatives thereof; wherein R may be hydrogen, or a saturated or unsaturated hydrocarbon that can be a straight aliphatic hydrocarbon, branched aliphatic hydrocarbon, aromatic hydrocarbon or substituted derivatives thereof, or cyclic hydrocarbon or substituted derivatives thereof; and wherein n may be a whole number less than or equal to 16. Bioavailability of the glycosidase inhibitor across said individual's blood-brain barrier is substantially better than N-butyl DNJ. For example, the glycosidase inhibitor may be N-nonyl DNJ.

A long chain N-alkyl derivative of DNJ (e.g., between five and 16 carbons, inclusive, in length) may be used as the compound of the present invention. More preferred are long chain N-alkyl derivatives between eight and 16 carbons, inclusive, in length. By comparative example, the unexpected advantage of using N-nonyl DNJ (i.e., the N-alkyl derivative of DNJ with a nine carbon chain) instead of N-butyl DNJ (i.e., the N-alkyl derivative of DNJ with a four carbon chain) to treat a brain tumor is demonstrated. Thus, short chain N-alkyl derivatives of DNJ (i.e., four carbons or less in length) may be used but are not preferred.

The compound can be administered to an individual affected by cancer, especially a solid tumor or other neoplasm. Anti-tumor activity is not necessarily related to other functions of the compound. Thus, while certain short chain N-alkyl derivatives of imino sugars (e.g., N-butyl DNJ) are potent inhibitors of the N-linked oligosaccharide processing enzymes, such as α -glucosidase I and α -glucosidase II (Saunier *et al.*, *J Biol Chem* 257: 14155-14161, 1982; Elbein, *Ann Rev Biochem* 56:497-534, 1987), some compounds of the present invention may exhibit substantially little or no inhibition of a glycosidase, especially in comparison with N-butyl DNJ.

Amino and imino compounds used as starting materials in the preparation of long chain N-alkylated compounds are commercially available (Sigma, St. Louis, Missouri, US; Cambridge Research Biochemicals, Norwich, Cheshire, UK; Toronto Research Chemicals, Ontario, CA) or can be prepared by known synthetic methods. Long chain N-alkylated compounds can be prepared by reductive alkylation of amino or imino compounds. For example, the amino or imino compound can be exposed to long chain aldehyde and reducing agent (e.g., sodium cyanoborohydride) to N-alkylate the amine. In particular, the compound can be a long chain N-alkylated imino sugar. The imino sugar can be, for example, deoxynorjirimycin (DNJ) or derivatives, enantiomers, or stereoisomers thereof. The compound can be prepared stereospecifically using a stereospecific amino or imino compound as a starting material. Alternatively, the compound can be purified out of a mixture of products after syn-

thesis. The compounds can be purified, for example, by crystallization or chromatographic methods.

The synthesis of a variety of imino sugars are also known in the art. For example, methods of synthesizing DNJ derivatives are known and are described, for example, in U.S. Patent Nos. 5,622,972, 5,200,523, 5,043,273, 4,994,572, 4,246,345, 4,266,025, 4,405,714, and 4,806,650, and U.S. patent application 07/851,818. Methods of synthesizing other imino sugar derivatives are known and are described, for example, in U.S. Patent Nos. 4,861,892, 4,894,388, 4,910,310, 4,996,329, 5,011,929, 5,013,842, 5,017,704, 5,580,884, 5,286,877, and 5,100,797. The substituents on the imino sugar can influence the efficacy of the compound as an anti-tumor agent and, additionally, can preferentially target the molecule to one organ rather than another.

The compounds can include protecting groups. Various protecting groups are well known. In general, the species of protecting group is not critical, provided that it is stable to the conditions of any subsequent reaction(s) on other positions of the compound and can be removed at the appropriate point without adversely affecting the remainder of the molecule. In addition, a protecting group may be substituted for another after substantive synthetic transformations are complete. Where a compound differs from a compound disclosed herein only in that one or more protecting groups of the disclosed compound has been substituted with a different protecting group, that compound is within the present invention. Further examples and conditions are found in *Protective Groups in Organic Chemistry* by T.W. Greene, 1st ed., 1981; Greene and Wuts, 2nd ed., 1991).

Compounds of the present invention may be used to produce a medicament or other pharmaceutical composition to treat a tumor or other neoplasm.

Compounds described herein may be used in the free amine form or in a pharmaceutically acceptable salt form. Pharmaceutical salts and methods for preparing salt forms are provided in Berge *et al.*, *J Pharm Sci* 66:1-18, 1977. Pharmaceutically acceptable salts can be preferred for compounds that are difficult to solubilize in the pharmaceutical composition (e.g., compounds having longer alkyl chains). A salt form is illustrated, for example, by the HCl salt of an amino derivative. For example, the compounds can be di- or tetra- acetates, propionates, butyrates, or isobutyrate. The compound can be a solvate.

The compounds may also be used in the form of prodrugs such as, for example, the 6-phosphorylated DNJ derivatives described in U.S. Patent Nos. 5,043,273 and 5,103,008.

Use of compositions which further comprise a pharmaceutically acceptable carrier and compositions which further comprise components useful for delivering the composition

to an individual are known in the art. Addition of such carriers and other components to the composition of the present invention is well within the level of skill in this art.

Pharmaceutical compositions that are useful in the present invention may be administered as an oral, ophthalmic, suppository, aerosol, topical, or other formulation. For example, it may be in the physical form of a solid, powder, tablet or lozenge, capsule, liquid or solution, gel, emulsion, suspension, syrup, or the like. In addition to the compound, such compositions may contain pharmaceutically-acceptable carriers and other ingredients known to facilitate administration and/or enhance uptake (*e.g.*, saline, dimethyl sulfoxide). Other formulations, such as nanoparticles, liposomes, and immunologically-based systems may also be used in accordance with the present invention. For a solid tumor or other neoplasm, the composition may be incorporated in a permeable matrix (*e.g.*, a bead or disk) placed adjacent to the tumor or other neoplasm for sustained, local release.

Pharmaceutical compositions may be administered by any known route. By way of example, the composition may be administered by a mucosal, pulmonary, topical, or other localized or systemic route (*e.g.*, enteral and parenteral). The term "parenteral" includes subcutaneous, intradermal, intramuscular, intravenous, intraarterial, intrathecal, and other injection or infusion techniques, without limitation.

These compositions may be administered according to the present invention in a single dose or in multiple doses which are administered at different times. Because the effect of the composition upon a tumor or other neoplasm may persist, the dosing regimen may be adjusted such that chemotherapy is promoted while the individual is otherwise minimally effected. By way of example, an individual may be administered a dose of the composition once per day, whereby growth of the tumor or other neoplasm is slowed or reduced for the entire or most of the day, while the individual's normal functions are inhibited for only a short period during the day.

Suitable choices in formulation, administration, and dosing can be made with the goals of achieving a favorable response in the individual with respect to the tumor or other neoplasm (*i.e.*, efficacy), and avoiding undue toxicity or other harm (*i.e.*, safety) thereto.

Compound of the present invention, or a pharmaceutical composition thereof, is administered to an individual in an amount effective to slow or reduce growth of a tumor or other neoplasm. The term "slow or reduce" refers to the detectable slowing of the time rate of change in size and/or reduction (*i.e.*, change in size) of growth of a tumor or other neoplasm. Tumor volume is understood to be a measure of size. The term "effective amount" refers to that amount of compound thereof necessary to achieve a therapeutic effect.

The term "treatment" refers to reducing or alleviating symptoms in an individual, preventing symptoms from worsening or progressing, and/or preventing disease in an individual who is free therefrom as well as slowing or reducing growth of a tumor or other neoplasm. For a given individual, improvement in a symptom, its worsening, regression, or progression may be determined by any objective or subjective measure. Efficacy of the treatment may be measured as an improvement in morbidity or mortality (e.g., lengthening of survival curve for a selected population). Treatment may also involve debulking the tumor or other neoplasm by surgical and/or radiation therapy, especially if performed prior to chemotherapy. Thus, combination therapy with one or more medical/surgical procedures and one or more other chemotherapeutic agents may be practiced with present invention. Prophylactic methods (e.g., preventing or reducing the incidence of relapse) are also considered treatment.

The amount which is administered to an individual is preferably an amount that does not induce toxic effects which outweigh the advantages which accompany its administration. For example, it is preferred that the effective amount used in the present invention does not substantively lower the treated individual's level of blood glucose. Further objectives of the present invention are to reduce in number, diminish in severity, and/or otherwise relieve suffering from the symptoms of the disease as compared to recognized standards of care. In addition to treatment of primary disease, the present invention may also be effective against metastatic disease.

A bolus administered over a short time once a day is a convenient dosing schedule. Alternatively, the effective daily dose may be divided into multiple doses for purposes of administration, for example, two to twelve doses per day. Dosage levels of active ingredients in a pharmaceutical composition can also be varied so as to achieve a transient or sustained concentration of the compound in an individual, especially in and around the tumor or other neoplasm, and to result in the desired therapeutic response. But it is also within the skill of the art to start doses of the compound at levels lower than required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. It will be understood that the specific dose level for any particular individual will depend on a variety of factors, including body weight, general health, diet, size and change in size of the tumor or other neoplasm, route and scheduling of administration, combination with one or more other drugs, and severity of disease.

The dose level selected for use in the present invention will depend on the bioavailability, activity, and stability of the compound, the route of administration, the severity of the disease being treated, and the condition and medical history of the individual in need of treat-

ment. It is contemplated that a daily dosage may be between about one microgram to about one gram, preferably from anywhere between about 10-50 mg and about 100-500 mg (*e.g.*, 25 to 250 mg), of the compound per kilogram body weight. Such quantities may be used in a unit dose (*i.e.*, a dose sufficient for a single use once to several times per day). The amount
5 of compound administered is dependent upon factors known to a person skilled in this art such as, for example, the molecular weight and hydrophobicity the compound, the route of administration, location and type of tumor or other neoplasm, and the like.

In accordance with the present invention, the individual's tumor or other neoplasm may be benign or malignant. For example, the individual's disease may be classified as an
10 adenoma, carcinoma, hepatoma, or sarcoma. In particular, the bioavailability of compounds of the present invention across the blood-brain barrier makes it advantageous to treat brain cancers (*e.g.*, astrocytomas, gliomas, meningiomas, neurinomas). Furthermore, the tumor or other neoplasm may be derived from different tissue types such as, for example, ectoderm, embryonic, endoderm, epithelium, and neuroectoderm, especially solid tissues.

15 The individual may be any animal or patient with cancer. Mammals, especially humans, may be treated by the present invention. Thus, both veterinary and medical treatments are envisioned.

The following examples are merely illustrative of the present invention and do not limit or restrict its practice.

20

EXAMPLE 1

The CT-2A brain tumor (Seyfried *et al.*, *Mol Chem Neuropathology* 17:147-167, 1992), was produced in C57BL/6J mice using the procedure of Zimmerman and Arnold (*Cancer Res* 1:919-938, 1941). Such brain tumors have been induced in mice with 20-
25 methylcholanthrene (MC) and used extensively as animal models for evaluating chemotherapies for individuals with a brain tumor (Shapiro *et al.*, *Cancer Res* 30:2401-2413, 1970; Crafts and Wilson, *Natl Cancer Inst Monogr* 46:11-17, 1977; Zimmerman, *Ann NY Acad Sci* 381:320-324, 1982; Schold and Bigner, In: Walker, *Oncology of the Nervous System*, Martinus Nijhoff, Boston, pp. 31-64, 1983).

30 These brain tumor models are ideally suited for these studies with imino sugars and other glycosidase inhibitors because they are grown in the natural syngeneic host and have relatively simple ganglioside compositions that remain stable in both *in vitro* and *in vivo* environments. This contrasts with human glioma models where ganglioside composition changes dramatically in response to the local environment and makes it difficult to interpret

results from such models; such problems are highlighted by ganglioside analysis in a xenograft model (Ecsedy *et al.*, *J Neurochem* 73:254-259, 1999). Thus, the 20-MC brain tumor model is ideally suited for evaluating the efficacy of novel chemotherapeutics.

We have discovered that N-nonyl-1,5-deoxy-1,5-imino-D-glucitol (N-nonyl DNJ) is considerably more effective in inhibiting tumor growth than another N-alkyl-DNJ species to which it is compared. Experimental results indicate that N-nonyl DNJ is more potent than N-butyl DNJ in reducing volume and growth rate of the CT-2A tumor grown in the flanks of mice injected with CT-2A. Figure 1 shows experimental data indicating that N-nonyl DNJ inhibited experimental brain tumor growth in mice more effectively than N-butyl DNJ even when administered at one-tenth the dose (50 mg/kg/day vs. 500 mg/kg/day). These doses of N-nonyl DNJ were equivalent in effectiveness to treatment with N-butyl DNJ at a dose of 2400 mg/kg/day. Thus, the present invention using long-chain alkyl compounds, especially long-chain N-alkyl DNJ derivatives, is unexpectedly efficacious as compared to short-chain alkyl compounds. Here, a suitable dose is about 50-100 mg/kg/day.

EXAMPLE 2

Six- to eight-week old male C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were inoculated subcutaneously in the flank with the CT-2A tumor line (Department of Biology, Boston College) using 0.1 cc nondissociated tumor chunks taken up to 0.2 cc with phosphate buffered saline (PBS), using an 18-gauge needle.

The following doses of N-nonyl DNJ or N-butyl DNJ, admixed with mouse chow, were administered to the control and inoculated individuals:

	Control	powdered food only	n=4
	N-nonyl DNJ	50 mg/kg/day	n=3
25	N-nonyl DNJ	500 mg/kg/day	n=6
	N-butyl DNJ	500 mg/kg/day	n=5
	N-butyl DNJ	2400 mg/kg/day	n=2

Tumor volume was measured every other day beginning with day 0 of treatment, from an initial tumor volume of about 20-45 mm³, for eight days. The average \pm SEM is reported for tumor volume.

Table 1. Control mice, tumor size (mm³)

Treatment Day	Mouse ID				Average Tumor Volume
	#25	#33	#50	#47	
0	27	30	35	38	33 ± 2.5
2	123	44	116	70	88 ± 19
4	281	135	214	255	221 ± 32
6	595	164	772	651	546 ± 132
8	1188	327	1566	1764	1211 ± 318
Ratio 8/0	44.0	11.0	44.7	46.4	36.7

Table 2. 50 mg N-nonyl DNJ-treated mice, tumor size (mm³)

Treatment Day	Mouse ID			Average Tumor Volume
	#22	#27	#35	
0	27	38	30	32 ± 3.3
2	53	44	63	53 ± 5.5
4	89	125	111	108 ± 10
6	149	158	289	199 ± 45
8	405	289	352	349 ± 34
Ratio: 8/0	15.0	7.6	11.7	10.9

5 Table 3. 500 mg N-nonyl DNJ-treated mice, tumor size (mm³)

Treatment Day	Mouse ID						Average Tumor Volume
	#21	#26	#38	#42	#48	#43	
0	38	40	23	36	23	30	32 ± 3.1
2	49	96	33	45	38	57	53 ± 9.3
4	128	232	53	78	102	131	121 ± 25
6	179	340	91	147	192	203	192 ± 34
8	357	442	150	378	384	430	357 ± 43
Ratio: 8/0	9.4	11.1	6.5	10.5	16.7	13.4	11.1

Table 4. 500 mg N-butyl DNJ-treated mice, tumor size (mm³)

Treatment Day	Mouse ID					Average Tumor Volume
	#36	#31	#40	#45	#49	
0	29	30	28	40	41	34 ± 2.8
2	69	56	41	45	95	61 ± 10
4	175	150	84	206	205	164 ± 23
6	243	206	247	275	351	265 ± 24
8	616	540	306	381	547	478 ± 58
Ratio: 8/0	21.2	18.0	10.9	9.5	13.3	14.1

Table 5. 2400 mg N-butyl DNJ-treated mice, tumor size (mm³)

Treatment Day	Mouse ID		Average Tumor Volume
	#32	#39	
0	42	48	45
2	69	143	106
4	195	149	172
6	252	255	254
8	346	332	339
Ratio: 8/0	8.2	6.9	7.5

- 5 Reduction in tumor size during treatment compared to controls after eight days:
 - a. N-nonyl DNJ (50 mg/kg/day): 71% reduction
 - b. N-nonyl DNJ (500 mg/kg/day): 71% reduction
 - c. N-butyl DNJ (500 mg/kg/day): 61% reduction
 - d. N-butyl DNJ (2400 mg/kg/day): 72% reduction
- 10 Reduction of tumor change in size during treatment compared to controls after eight days:
 - a. N-nonyl DNJ (50 mg/kg/day): 70% reduction
 - b. N-nonyl DNJ (500 mg/kg/day): 70% reduction
 - c. N-butyl DNJ (500 mg/kg/day): 62% reduction
 - d. N-butyl DNJ (2400 mg/kg/day): 80% reduction

EXAMPLE 3

Six- to eight-week old male C57BLJ/6J mice were inoculated subcutaneously in the flank with the CT-2A tumor line using 0.1 cc nondissociated tumor chunks taken up to 0.2 cc with PBS, and an 18-gauge needle.

5	Control a	Powdered food only	n=3
	Control b	Powdered food only, no tumor	n=3
	N-nonyl DNJ	50 mg/kg/day	n=3
	N-nonyl DNJ	500 mg/kg/day	n=3
	N-butyl DNJ	500 mg/kg/day	n=3
10	N-butyl DNJ	2400 mg/kg/day	n=2

The average \pm SEM for blood glucose concentration is reported.

Table 6. Trinder assay for blood glucose levels

Control a (+CT-2A)		Control b (-CT-2A)		N-nonyl DNJ (50 mg/kg/day)	
Mouse ID	mM/L glucose	Mouse ID	mM/L glucose	Mouse ID	mM/L glucose
#25	11.5	#1	12.4	#22	16.7
#46	11.0	#2	15.7	#24	13.6
#47	10.7	#3	11.6	#27	9.9
11.0 \pm 0.2		13.2 \pm 1.3		13.4 \pm 2.0	

N-nonyl DNJ (500 mg/kg/day)		N-butyl DNJ (500 mg/kg/day)		N-butyl DNJ (2400 mg/kg/day)	
Mouse ID	mM/L glucose	Mouse ID	mM/L glucose	Mouse ID	mM/L glucose
#21	12.6	#36	9.7	#28	6.7
#37	11.3	#40	9.5	#32	6.9
#48	9.0	#45	7.4		
11.0 \pm 1.1		8.9 \pm 0.7		6.8 \pm 0.1	

15

Level of blood glucose is normal or higher with N-nonyl DNJ compared to N-butyl DNJ

- a. CT-2A-bearing controls had 16% lower blood glucose levels than non-tumor-bearing controls.
- b. N-nonyl DNJ (50 mg/kg/day)-treated mice had blood glucose levels 17% higher than CT-2A-bearing controls.
- c. N-nonyl DNJ (500 mg/kg/day)-treated mice had blood glucose levels equal to the CT-2A-bearing controls.
- d. N-butyl DNJ (500 mg/kg/day)-treated mice had blood glucose levels 19.8% lower than CT-2A-bearing controls.
- e. N-butyl DNJ (2400 mg/kg/day)-treated mice had blood glucose levels 39% lower than CT-2A-bearing controls.

EXAMPLE 4

The CT-2A tumor line was intracerebrally implanted in six- to eight-week old male C57BL/6J mice by the method of Zimmerman and Arnold (*Cancer Res* 1:919-938, 1941). Treatment with N-nonyl DNJ (330 mg/kg/day admixed into mouse chow) was initiated 48 hr post-implantation; controls were not treated. The average \pm SEM is reported for tumor dry weight after nine days of treatment.

Table 7. N-nonyl DNJ inhibits a tumor implanted in the brain

gm Control	gm Treated
46.7	11.1
51.8	9.0
11.2	22.9
27.0	7.3
59.2	16.5
16.9	5.8
21.9	6.8
14.5	16.2
42.2	9.7
31.5	10.1
	7.0
	7.4
32.3 \pm 5.3	10.8 \pm 1.5

This difference in tumor size is highly significant ($p < 0.001$ by two-tailed Students *t* test).

EXAMPLE 5

Healthy six- to eight-week old male C57BL/6J mice (*i.e.*, not tumor-bearing) were fed either N-butyl DNJ or N-nonyl DNJ (330 mg/kg/day admixed into mouse chow) for 60 days. Compound levels in various tissues were quantitated by separation using a high-performance anion-exchange column coupled with pulsed amperometric detection (HPAE-PAD) from Dionex (Sunnyvale, California, US). Measurements were made as shown in Table 8.

The uptake of N-butyl DNJ in brain and liver was much less than that of N-nonyl DNJ. Only trace quantities of N-butyl DNJ was found in the brain on days 1 and 3, and was undetectable in the brain thereafter. The quantity of N-butyl DNJ in the liver was higher than in brain, but only ranged from 0.3 $\mu\text{g/gm}$ to 1.1 $\mu\text{g/gm}$ (wet weight) when measured over the 60 days.

Table 8. Distribution of N-nonyl DNJ in various body parts

Treatment Day	$\mu\text{g/gm}$ Wet Weight			
	Liver	Testes	Brain	Serum
1	3.4	1.9	1.6	5.9
3	3.0	5.3	3.1	4.3
5	7.2	2.1	2.2	1.8
7	4.5	4.2	3.6	24.0
9	8.8	7.7	3.5	12.8
14	8.8	6.0	3.1	11.9
30	4.2	3.4	1.5	12.8
60	4.1	8.1	1.8	13.3

15

To provide a possible mechanism by which the present invention may operate, but without intending to be bound by any hypothesis, the greater effectiveness of N-nonyl DNJ as compared to N-butyl DNJ in inhibiting tumor growth may be linked to its hydrophobic nature. This may result in a more favorable biodistribution for treatment of cancer, especially brain tumors.

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Furthermore, these experiments demonstrate the effect on glucose levels in the treated individuals that is associated with the impact of the N-alkyl-1,5-deoxy-1,5-imino-D-glucitol compounds. N-nonyl DNJ treated tumor-bearing individuals maintained blood glucose levels equal to or greater than that of tumor-bearing controls, while N-butyl DNJ-treated tumor-bearing individuals exhibited lower blood glucose levels. Without intending to be bound by any hypothesis, the present invention have exert a direct effect on a tumor or other neoplasm instead of simply reducing a source of energy for those cells.

N-alkyl-DNJ compounds may or may not affect tumor growth by inhibiting one or more glycolipid biosynthesis mechanisms, glucosidase-dependent cell processes, angiogenesis, and/or tumorigenesis. It is believed the effectiveness shown above is generally applicable to the treatment of all tumors dependent on the same processes to invade and expand from a local site. For that reason, the present invention is not limited to the treatment of tumors of the brain or nervous system.

All references (*e.g.*, publications, books, patents and patent applications) cited above are indicative of the level of skill in the art and are incorporated by reference therein.

All modifications which come within the meaning of the claims and the range of their legal equivalents are to be embraced within their scope. In particular, "comprising" allows the inclusion of other elements in the claim, "comprising essentially of" allows the inclusion of other elements in the claim that do not materially affect operation of the present invention, and no particular relationship between or among elements of a claim is meant unless such limitation is explicitly recited (*e.g.*, arrangement of components in a product claim, order of steps in a method claim).

From the foregoing, it would be apparent to a person of skill in this art that the present invention can be embodied in other specific forms without departing from its spirit or essential characteristics. The described embodiments should be considered only as illustrative, not restrictive, because the scope of the legal protection provided for the present invention will be indicated by the appended claims rather than by the foregoing description.

WE CLAIM:

1. Use of an imino sugar or pharmaceutically acceptable salt thereof to treat an individual with a tumor or other neoplasm such that growth of said tumor or other neoplasm in said treated individual is at least slowed or reduced compared to growth prior to treatment, wherein said imino sugar is not N-butyl-1,5-deoxy-1,5-imino-D-glucitol (N-butyl DNJ).
2. Use according to claim 1 wherein said imino sugar is a galactoside analog.
3. Use according to claim 1 wherein said imino sugar has an N-alkyl chain of at least five carbons.
4. Use according to claim 3 wherein said imino sugar contains at least one C₅-C₁₆ substituent.
5. Use according to claim 1 wherein bioavailability of said imino sugar across said treated individual's blood-brain barrier is substantially better than N-butyl DNJ.
6. Use according to claim 5 wherein said imino sugar is N-nonyl-1,5-deoxy-1,5-imino-D-glucitol (N-nonyl DNJ).
7. Use according to claim 1 wherein said tumor or other neoplasm is benign.
8. Use according to claim 1 wherein said tumor or other neoplasm is malignant.
9. Use according to claim 1 wherein said tumor or other neoplasm is selected from the group consisting of adenomas, carcinomas, hepatomas, and sarcomas.
10. Use according to claim 1 wherein said tumor or other neoplasm is brain cancer.
11. Use according to claim 1 wherein said brain cancer is selected from the group consisting of astrocytomas, gliomas, meningiomas, and neurinomas.
12. Use according to claim 1 wherein said tumor or other neoplasm is derived from tissue selected from the group consisting of ectoderm, embryonic, endoderm, epithelium, and neuroectoderm.

13. Use according to any one of claims 1-12 wherein treatment is administered by an enteral route.

14. Use according to any one of claims 1-12 wherein treatment is administered by a parenteral route.

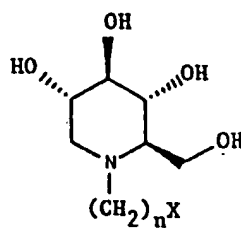
15. Use according to any one of claims 1-12 wherein said individual is a mammal.

16. Use according to claim 15 wherein said mammal is a human.

17. Use according to any one of claims 1-12 wherein said treatment does not lower said individual's level of blood glucose.

18. Use of 1-deoxynojirimycin (DNJ) or a derivative thereof to treat an individual with a tumor or other neoplasm such that growth of said tumor or other neoplasm in said treated individual is at least slowed or reduced compared to growth prior to treatment, wherein said DNJ derivative is not N-butyl-1,5-deoxy-1,5-imino-D-glucitol (N-butyl DNJ).

19. Use according to claim 18 wherein a DNJ derivative according to Formula A is used,



A

X being selected from the group consisting of unsaturated straight aliphatic hydrocarbons, saturated and unsaturated branched aliphatic hydrocarbons, aromatic hydrocarbons and substituted derivatives thereof, cyclic hydrocarbons and substituted derivatives thereof, -O-Y, -S-Y, -Y-OH, -Y-NH₂, -Y-COOH; -Y-CON⁺-R, and -Y-COO⁻-R;

Y being a saturated or unsaturated hydrocarbon selected from the group consisting of straight aliphatic hydrocarbons, branched aliphatic hydrocarbons, aromatic hydrocarbons and substituted derivatives thereof, and cyclic hydrocarbons and substituted derivatives thereof;

R being hydrogen or a saturated or unsaturated hydrocarbon selected from the group consisting of straight aliphatic hydrocarbons, branched aliphatic hydrocarbons, aromatic hydrocarbons and substituted derivatives thereof, cyclic hydrocarbons and substituted derivatives thereof; and

n is a whole number less than or equal to 16.

20. Use according to claim 19 wherein said derivative has an alkyl chain of at least five carbons.

21. Use according to claim 20 wherein said derivative is a C₅-C₁₆ N-alkyl derivative of DNJ.

22. Use according to claim 19 wherein bioavailability of said derivative across said treated individual's blood-brain barrier is substantially better than N-butyl DNJ.

23. Use according to claim 22 wherein said derivative is N-nonyl DNJ.

24. Use according to claim 19 wherein said tumor or other neoplasm is benign.

25. Use according to claim 19 wherein said tumor or other neoplasm is malignant.

26. Use according to claim 19 wherein said tumor or other neoplasm is selected from the group consisting of adenomas, carcinomas, hepatomas, and sarcomas.

27. Use according to claim 19 wherein said tumor or other neoplasm is brain cancer.

28. Use according to claim 27 wherein said brain cancer is selected from the group consisting of astrocytomas, gliomas, meningiomas, and neurinomas.

29. Use according to claim 19 wherein said tumor or other neoplasm is derived from tissue selected from the group consisting of ectoderm, embryonic, endoderm, epithelium, and neuroectoderm.

30. Use according to any one of claims 18-29 wherein treatment is administered by an enteral route.

31. Use according to any one of claims 18-29 wherein treatment is administered by a parenteral route.

32. Use according to any one of claims 18-29 wherein said individual is a mammal.

33. Use according to claim 32 wherein said mammal is a human.

34. Use according to any one of claims 18-29 wherein said treatment does not lower said individual's level of blood glucose.

35. Use of a glycosidase inhibitor or pharmaceutically acceptable salt thereof to treat an individual with a tumor or other neoplasm such that growth of said tumor or other neoplasm is at least slowed or reduced in said treated individual compared to growth prior to treatment, wherein said glycosidase inhibitor is not N-butyl-1,5-deoxy-1,5-imino-D-glucitol (N-butyl DNJ).

36. Use according to claim 35 wherein bioavailability of said glycosidase inhibitor across said individual's blood-brain barrier is substantially better than N-butyl DNJ.

37. Use according to any one of claims 35-36 wherein said treatment does not lower said individual's level of blood glucose.

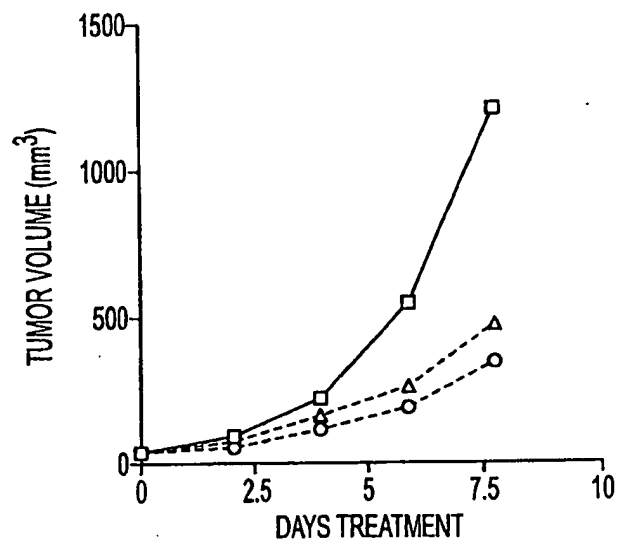


FIGURE 1

INTERNATIONAL SEARCH REPORT

b. National Application No

PCT/US 00/06933

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/445 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

CHEM ABS Data, BIOSIS, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 99 24401 A (G. . SEARLE & CO.) 20 May 1999 (1999-05-20) claims 1-27	1-35
X	EP 0 328 111 A (MEIJI SEIKA KABUSHIKI KAISHA) 16 August 1989 (1989-08-16) claim 1	1-5, 7-22, 24-37
X	US 4 837 237 A (L. R. ROHRSCHEIDER ET AL) 6 June 1989 (1989-06-06) claims 1-13	1,2,5, 7-18, 30-37
	-/-	

☒ Further documents are listed in the continuation of box C.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 15, no. 92 (C-811), 6 March 1991 (1991-03-06) & JP 02 306962 A (MEIJI SEIKA KAISHA LTD), 20 December 1990 (1990-12-20) abstract	1-5, 7-22, 24-37
X	PATENT ABSTRACTS OF JAPAN vol. 16, no. 544 (C-1004), 13 November 1992 (1992-11-13) & JP 04 208264 A (TSUMURA & CO), 29 July 1992 (1992-07-29) abstract	35-37
X	PATENT ABSTRACTS OF JAPAN vol. 1998, no. 06, 30 April 1998 (1998-04-30) & JP 10 045588 A (ISHIHARA SANGYO KAISHA LTD), 17 February 1998 (1998-02-17) abstract	35-37
X	PATENT ABSTRACTS OF JAPAN vol. 1997, no. 05, 30 May 1997 (1997-05-30) & JP 09 003090 A (SANKYO CO LTD), 7 January 1997 (1997-01-07) abstract	35-37
X	PATENT ABSTRACTS OF JAPAN vol. 1996, no. 09, 30 September 1996 (1996-09-30) & JP 08 134091 A (SANKYO CO LTD), 28 May 1996 (1996-05-28) abstract	35-37
X	PATENT ABSTRACTS OF JAPAN vol. 1996, no. 07, 31 July 1996 (1996-07-31) & JP 08 059646 A (ISHIHARA SANGYO KAISHA LTD), 5 March 1996 (1996-03-05) abstract	35-37
X	PATENT ABSTRACTS OF JAPAN vol. 1995, no. 11, 26 December 1995 (1995-12-26) & JP 07 196490 A (TERUMO CORP; OTHERS: 01), 1 August 1995 (1995-08-01) abstract	35-37

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9924401 A	20-05-1999	AU 1297399 A	31-05-1999
EP 328111 A	16-08-1989	JP 1207235 A	21-08-1989
		JP 1918084 C	07-04-1995
		JP 6043306 B	08-06-1994
		JP 1265025 A	23-10-1989
		JP 1991228 C	22-11-1995
		JP 7008793 B	01-02-1995
		JP 1268635 A	26-10-1989
		JP 1991230 C	22-11-1995
		JP 7008794 B	01-02-1995
		JP 1313425 A	18-12-1989
		JP 1313426 A	18-12-1989
		JP 2038320 C	28-03-1996
		JP 7068215 B	26-07-1995
		DE 68929141 D	24-02-2000
		US 4985445 A	15-01-1991
		US 5250545 A	05-10-1993
US 4837237 A	06-06-1989	NONE	
JP 02306962 A	20-12-1990	NONE	
JP 04208264 A	29-07-1992	NONE	
JP 10045588 A	17-02-1998	NONE	
JP 09003090 A	07-01-1997	NONE	
JP 08134091 A	28-05-1996	NONE	
JP 08059646 A	05-03-1996	NONE	
JP 07196490 A	01-08-1995	NONE	



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(74) Agents: LEE, Nicholas, John et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).			
(54) Title: USE OF GLUCOSYLCERAMIDE SYNTHESIS INHIBITORS IN THERAPY			
(57) Abstract			
<p>The present invention relates to the treatment of conditions such as Niemann-Pick C storage disease, Alzheimer's disease, epilepsy, stroke and Parkinson's disease, and in particular to the use of inhibitors of glucosylceramide synthesis in such treatment. Preferred inhibitors of glucosylceramide synthesis are imino sugar-structured, and include N-butyldeoxynojirimycin (NB-DNJ), N-butyldeoxygalactonojirimycin (NB-DGJ) and N-nonyldeoxynojirimycin (NN-DNJ).</p>			

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USE OF GLUCOSYLCERAMIDE SYNTHESIS INHIBITORS IN THERAPY

The present invention provides the use of inhibitors of glycolipid synthesis in the manufacture of medicaments for use in the treatment of conditions such as Niemann-Pick C storage disease, Alzheimer's disease, epilepsy, stroke and Parkinson's disease. In particular, the use of N-butyldeoxynojirimycin is provided.

Niemann-Pick Type C (NPC) disease, which is also known as Niemann-Pick disease with cholesterol esterification block, is an autosomal recessive storage disorder of cholesterol metabolism. NPC patients generally appear normal for the first few years of life. However, organomegaly of the liver and spleen soon emerge, and may result in jaundice or other symptoms of dysfunction. NPC patients also gradually develop neurologic abnormalities such as ataxia, tremors, seizures, and loss of speech, cognitive and motor skills, and difficulty with upward and downward eye movements. Impairment progresses, particularly resulting from increasing neural degeneration, and death usually occurs by 5-15 years of age.

Vanier *et al.* (1991) reported that Niemann-Pick Type C is heterogeneous, suggesting the possibility that more than one genetic mutation gives rise to the disease. Molecular studies recently substantiated this possibility. A gene most commonly mutated in Niemann-Pick Type C patients has been identified as NPC1 and mapped to 18q11-q12 (Carstea *et al.*, 1997). The NPC1 gene encodes a protein of 1,278 amino acids, and bears some sequence homology to the putative sterol-sensing regions of SREBP cleavage-activating protein and 3-hydroxy-3-methylglutaryl coenzyme A reductase (Carstea *et al.*, 1997). A specific function for the NPC1 gene product is unknown at this time, although biochemical studies are suggestive that NPC1 gene mutations somehow disturbs cholesterol metabolism. For example, NPC cells are blocked in cholesterol

esterification, but also do not effectively translocate cholesterol from lysosomes to other intracellular organelles (Pentchev *et al.* 1985, Sokol *et al.*, 1988).

Evidence for a second possible gene mutated in Niemann-Pick type C has been described, although it has not yet been identified (Steinberg *et al.*, 1994). Patients with NPC1 mutations have been subclassified as having Niemann-Pick type C1 disease, while patients with other mutated gene(s) as having Niemann-Pick type C2 disease. There is no known difference between the clinical courses of type C1 and C2 patients, and they appear to respond in the same way to disease treatments. In addition, the C1/C2 subclassification is not universally applied. Therefore, Niemann-Pick Type C diseases originating from NPC1 or other gene mutations are collectively referred to as NPC here.

Biochemical findings for NPC patients show a marked accumulation of cholesterol in the liver and spleen. The liver and spleen show elevated sphingomyelin levels. However, sphingomyelinase activity remains normal in these tissues. This finding distinguishes NPC from Niemann-Pick Types A and B diseases which are caused by lysosomal sphingomyelinase mutations, and so present with markedly reduced levels of this enzyme.

In addition to the liver and the spleen, other cells of NPC patients store cholesterol as well. For example, bone marrow cells take on a characteristic foamy appearance due to the presence of large numbers of storage inclusions, while eye and skin cells typically are less affected. Neuronal cells store some cholesterol, although glycolipid accumulation, particularly GM2 ganglioside, predominates.

There is as yet no accepted treatment for NPC disease. Given the observations supporting NPC disease's origin in a cholesterol metabolism defect, most treatment attempts have focused on reducing cholesterol storage (Sylvain *et al.*, 1994, *Pediatr.*

Neurol. 10:228-32, Patterson *et al*, 1993, *Neurology*, 43:61-4). However, restricting cholesterol intake or treating patients with a range of cholesterol-lowering drugs has had puzzlingly little effect on the tissue storage levels of this material, and no apparent effect on the disease's progress.

5

The perception in the art is that the glycolipid accumulation component of NPC disease is a secondary effect of the cholesterol metabolism defect component (see for example Chapter 85 in *The Metabolic and Molecular Bases of Inherited Disease*, 7th edition, McGraw-Hill Inc, New York, pp 2625-2639 (1995), Loftus *et al*, 1997, *Science*, 277: 232-235). Thus, until now, little attention has focussed on treating this component of the disease.

Affected neuronal cells in NPC patients undergo morphologic changes including the development of fibrillar tangles that are structurally similar to those seen in neurodegenerative disorders such as Alzheimer's disease and tuberous sclerosis. The age of onset and the rapidity of neuronal deterioration in NPC patients can vary considerably. The mechanism underlying these neurologic changes is unknown. It has been proposed that elevated levels of GM2, such as that seen in NPC patient neurons, may induce ectopic dendritic proliferation and meganeurite formation (Goodman and Walkley (1996) *Brain Res Dev Brain Res* 93:162-71), and dendritogenesis and neuron changes correlate well with disease severity in a feline model of NPC (March *et al*, 1997).

The imino sugar N-butyldeoxynojirimycin (NB-DNJ) is a potent inhibitor of alpha-glucosidase 1 (involved in N-glycan synthesis), and an even more potent inhibitor of glucosylceramide glucosyltransferase. NB-DNJ is currently undergoing clinical trials as a treatment for Gaucher and Fabry diseases, glycolipid storage disorders resulting from mutations in glucocerebrosidase and alpha-galactosidase A, respectively (see Figure 1 of the accompanying drawings). The rationale underlying these clinical trials is based on

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the observation that cells treated with NB-DNJ produce markedly reduced glucosylceramide levels because of the molecule's inhibition of glucosylceramide synthesis (see Figure 1 of the accompanying drawings). Thus, the clinical trials are determining whether patient health benefits could be achieved by balancing a NB-DNJ induced decrease in the rate of glucosylceramide synthesis against the impaired rate of glycolipid clearance seen in Gaucher and Fabry disease patients.

We have now found that neuronal glycolipid storage seen in NPC patients, for instance, may also be reduced by NB-DNJ treatment. As demonstrated herein, NB-DNJ markedly reduces clinical and pathological symptoms in feline and murine models of NPC.

Thus, in a first aspect, the present invention provides the use of an inhibitor of glucosylceramide synthesis in the manufacture of a medicament for use in the treatment of Niemann-Pick type C disease.

In the context of the present invention, the term "inhibitor" includes molecules such as N-butyldeoxynojirimycin, N-butyldeoxygalactonojirimycin, N-nonyldeoxynojirimycin and other imino sugar-structured inhibitors of glucosylceramide synthesis. However, in addition, it also includes other inhibitors of glucosylceramide synthesis, including agents such as 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol and structurally related analogues thereof. Furthermore, inhibition can also be achieved by the use of genetic approaches, based on the introduction of nucleic acid coding for proteins or peptides capable of inhibiting glucosylceramide synthesis or antisense sequences or catalytic RNA capable of interfering with the expression of enzymes responsible for glucosylceramide synthesis (e.g. glucosylceramide synthase). A combination of any of the above approaches can be used.

In a second aspect, the present invention provides the use of N-butyldeoxynojirimycin in the manufacture of a medicament for use in the treatment of Niemann-Pick type C disease.

5 In a third aspect, the present invention provides the use of an agent capable of increasing the rate of neuronal glycolipid degradation in the manufacture of a medicament for use in the treatment of Niemann-Pick type C disease. Examples of such agents include enzymes which degrade neuronal glycolipids, e.g. lysosomal hexoseaminidases, galactosidases, sialidases and glucosylceramide glucosidase, and molecules which increase the activity of
10 such enzyme. In addition, the agent could comprise a nucleic acid sequence (DNA or RNA) which codes for the enzymes mentioned above, i.e. such sequences could be introduced to increase natural production of such enzymes.

Lipid metabolism also plays a critical role in other neuronal disorders, such as
15 Alzheimer's disease and epilepsy. As mentioned above, NPC patient neurons present with fibrillar tangles reminiscent of the morphology seen in Alzheimer's disease. Interestingly, GM1 ganglioside binding by amyloid beta-protein induces conformational changes that support its formation of fibrous polymers, and the fibrillar deposition of this protein is an early event in Alzheimer's disease (Yanagisawa *et al* (1995) *Nat Med*
20 1:1062-6, Choo-Smith *et al* (1997) *Biol Chem* 272:22987-90). Thus, decreasing GM1 synthesis with agents such as NB-DNJ could inhibit the fibre formation seen in Alzheimer's disease.

Thus, in a fourth aspect, the present invention provides the use of an inhibitor of
25 glucosylceramide synthesis in the treatment of Alzheimer's disease.

Thus, in a fifth aspect, the present invention provides the use of an inhibitor of glucosylceramide synthesis in the treatment of epilepsy.

In a sixth aspect, the present invention provides the use of an agent capable of increasing the rate of neuronal glycolipid degradation in the manufacture of a medicament for use in the treatment of Alzheimer's disease or epilepsy.

5

In contrast, preliminary clinical trials have shown that neurodegenerative processes seen with Parkinson's disease, stroke and spinal cord injuries seem to improve by treating patients with GM1 ganglioside (Alter (1998) *Ann N Y Acad Sci* 845:391-4011; Schneider (1998) *Ann N Y Acad Sci* 845:363-73; Geisler (1998) *Ann N Y Acad Sci* 845: 374-81). It is possible that co-administering glucosylceramide synthesis inhibitors would provide the clinician greater control over this treatment course. Inhibitors like NB-DNJ would limit patient-specific inconsistencies by blocking their neuronal glycolipid synthesis. In addition, inhibiting glucosylceramide synthesis would limit the metabolism of administered glycolipids into other, perhaps unproductive, forms. Thus, the ability to modulate glucosylceramide synthesis with inhibitors such as NB-DNJ may be useful in treatment of a wide variety of neuronal disorders.

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According to an eighth aspect of the present invention, there is provided the use of an inhibitor of glucosylceramide synthesis in the production of a medicament for the treatment of a condition treatable by the administration of a ganglioside such as GM1 ganglioside. Examples of such conditions are Parkinson's disease, stroke and spinal cord injuries.

25

The medicament may further comprise a ganglioside such as GM1 ganglioside.

The invention also provides, in a ninth aspect, a product comprising an inhibitor of glucosylceramide synthesis and a ganglioside (preferably GM1 ganglioside) as a

combined preparation for simultaneous, sequential or separate use in the treatment of a condition treatable by the administration of a ganglioside, such as GM1 ganglioside.

Methods and processes for the production of N-butyldeoxynojirimycin can be found for example in US-A-4182767, EP-B-0012278, EP-A-0624652, US-A-4266025, US-A-4405714 and US-A-5151519 for example.

In other aspects, the present invention provides:

- 10 (a) a method for the treatment of Niemann-Pick type C disease which comprises administering to a subject in need thereof a therapeutically effective amount of a glucosylceramide synthesis inhibitor;
- (b) a method for the treatment of Niemann-Pick type C disease which comprises administering to a subject in need thereof a therapeutically effective amount of N-
15 butyldeoxynojirimycin;
- (c) a method for the treatment of Niemann-Pick type C disease which comprises administering to a subject in need thereof a therapeutically effective amount of an agent capable of increasing the rate of degradation of neuronal glycolipids;
- (d) a method for the treatment of Alzheimer's disease or epilepsy which comprises
20 administering to a subject in need thereof a therapeutically effective amount of a glucosylceramide synthesis inhibitor;
- (e) a method for the treatment of Alzheimer's disease or epilepsy which comprises administering to a subject in need thereof a therapeutically effective amount of N-
butyldeoxynojirimycin;
- 25 (f) a method for the treatment of Alzheimer's disease or epilepsy which comprises administering to a subject in need thereof a therapeutically effective amount of an agent capable of increasing the rate of degradation of neuronal glycolipids;

- (g) a method for the treatment of a condition treatable by the administration of a ganglioside, such as GM1 ganglioside, which comprises administering to a subject in need thereof a therapeutically effective amount of a glucosylceramide synthesis inhibitor;
- 5 (h) a method for the treatment of a condition treatable by the administration of a ganglioside such as GM1 ganglioside which comprises administering to a subject in need thereof a therapeutically effective amount of N-butyldeoxynojirimycin;
- (i) a method for the treatment of a condition treatable by the administration of a ganglioside such as GM1 ganglioside which comprises administering to a subject
- 10 in need thereof a therapeutically effective amount of an agent capable of increasing the rate of degradation of neuronal glycolipids.

The medicaments described herein and which are also for use in the methods provided herein, may include one or more of the following: preserving agents, solubilising agents,

15 stabilising agents, wetting agents, emulsifiers, sweeteners, colorants, odourants, salts, buffers, coating agents or antioxidants. They may also contain therapeutically active agents in addition to the compounds and/or agents described herein.

Routes of Administration

20 The medicaments may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intramuscular, intravenous or intradermal) route. Such a composition may be prepared by any method known in the art of pharmacy, for example by admixing the active ingredient with a carrier

25 under sterile conditions.

Various routes of administration will now be considered in greater detail:

(i) Oral Administration

Medicaments adapted for oral administration may be provided as capsules or tablets; as powders or granules; as solutions, syrups or suspensions (in aqueous or non-aqueous liquids); as edible foams or whips; or as emulsions.

- 5 Tablets or hard gelatine capsules may comprise lactose, maize starch or derivatives thereof, stearic acid or salts thereof.

Soft gelatine capsules may comprise vegetable oils, waxes, fats, semi-solid, or liquid polyols etc.

10

Solutions and syrups may comprise water, polyols and sugars. For the preparation of suspensions oils (e.g. vegetable oils) may be used to provide oil-in-water or water-in-oil suspensions.

- 15 (ii) *Transdermal Administration*

Medicaments adapted for transdermal administration may be provided as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. For example, the active ingredient may be delivered from the patch by iontophoresis (Iontophoresis is described in *Pharmaceutical Research*, 3(6):318 (1986)).

20

- (iii) *Topical Administration*

Medicaments adapted for topical administration may be provided as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols or oils.

- 25 For infections of the eye or other external tissues, for example mouth and skin, a topical ointment or cream is preferably used. When formulated in an ointment, the active ingredient may be employed with either a paraffinic or a water-miscible ointment base.

Alternatively, the active ingredient may be formulated in a cream with an oil-in-water base or a water-in-oil base.

5 Medicaments adapted for topical administration to the eye include eye drops. Here the active ingredient can be dissolved or suspended in a suitable carrier, e.g. in an aqueous solvent.

10 Medicaments adapted for topical administration in the mouth include lozenges, pastilles and mouthwashes.

(iv) *Rectal Administration*

Medicaments adapted for rectal administration may be provided as suppositories or enemas.

(v) *Nasal Administration*

15 Medicaments adapted for nasal administration which use solid carriers include a coarse powder (e.g. having a particle size in the range of 20 to 500 microns). This can be administered in the manner in which snuff is taken, i.e. by rapid inhalation through the nose from a container of powder held close to the nose.

20 Compositions adopted for nasal administration which use liquid carriers include nasal sprays or nasal drops. These may comprise aqueous or oil solutions of the active ingredient.

25 Medicaments adapted for administration by inhalation include fine particle dusts or mists, which may be generated by means of various types of apparatus, e.g. pressurised aerosols, nebulisers or insufflators. Such apparatus can be constructed so as to provide predetermined dosages of the active ingredient.

(vi) *Vaginal Administration*

Medicaments adapted for vaginal administration may be provided as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

(vii) Parenteral Administration

- 5 Medicaments adapted for parenteral administration include aqueous and non-aqueous sterile injectable solutions or suspensions. These may contain antioxidants, buffers, bacteriostats and solutes which render the compositions substantially isotonic with the blood of an intended recipient. Other components which may be present in such compositions include water, alcohols, polyols, glycerine and vegetable oils, for example. Compositions adapted
10 for parenteral administration may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of a sterile liquid carrier, e.g. sterile water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

15

Dosages

- Dosages will be readily determinable by routine trials, and will be under the control of the physician or clinician. The guiding principle for determining a suitable dose will be delivery of a suitably efficacious but non-toxic, or acceptably toxic, amount
20 of material. For NB-DNJ or a similar compound, a daily dosage for an adult could be expected to be in the range of from 1 mg to 2 g of active agent, and may be in the range of from 100 to 800 mg, or 300 to 600 mg. The dosage may be administered in a single daily dose or alternatively in two, three or more doses during the day.

- 25 Preferred features of each aspect of the invention are as for each of the other aspects. *mutatis mutandis*.

In the accompanying drawings:

Figure 1 is a schematic representation of the synthesis and degradation of glucosylceramide-containing glycolipids. Examples of genetic diseases resulting from a defect in one of the enzymes required for glycolipid degradation are indicated. The enzyme reaction inhibited by N-butyldeoxynojirimycin to decrease the synthesis of glucosylceramide-containing glycolipids is also shown.

The invention will now be described with reference to the following examples, which should not in any way be construed as limiting the scope of the invention.

EXAMPLES

Example 1 – Inhibition of clinical and pathological symptoms in a feline model of NPC

A domestic cat model of Niemann-Pick C has been described that demonstrates the disorder's characteristic liver storage of cholesterol, glucosylceramide, lactosylceramide and phospholipids, and neuronal storage of GM2 and GM3 gangliosides (Lowenthal *et al* (1990) *Acta Neuropathol. (Berl)* 81:189-197). A breeding colony for this animal model of NPC is being maintained to study the disease and its potential treatments (Brown *et al* (1996) *J. Inherit Metab. Dis.* 19:319-330;). NPC cats exhibit clinical signs of the disease beginning around 2-3 months with ataxia and titubation, and progress to severe ataxia and death by around 10-12 months.

From seven feline NPC carrier litter mates, normal and NPC-affected male and female cats were selected for the study. The affected female and unaffected male began treatment with NB-DNJ at 1200 mg/kg/day. This administration level proved to be acutely hepatotoxic to the cats, so the treatments quickly had to be ceased. During a

brief recovery period for these animals, an unrelated normal cat was treated to determine the maximum tolerated dose for NB-DNJ in this species. Based on this dose-ranging work, the NPC-affected and unaffected litter mates were restarted with NB-DNJ at 50 mg/kg/day. Over the following weeks, the administration level was increased to 150 mg/kg/day. This dose, too, proved to be hepatotoxic, so the administration level was maintained thereafter at 100 mg/kg/day. Except for brief intervals when the treatments were withheld because of transient appetite loss, the NB-DNJ dosages were continued for about three months. On this date, the animals were sacrificed for histologic and lipid analyses.

10

The following sections highlight the medical and neurologic findings for the study animals.

Cat number: S219

15 *Status: Normal, non-treated*

Date of Birth: 4 Nov., 1997

Gender: Male

This cat had an unremarkable developmental course throughout the treatment period, with normal behaviour, mobility and reflexes. He also underwent a normal weight gain, reaching about 3.6 kg by the end of the treatment period. He was not subjected to neurologic assessments during the treatment period.

20

Cat number: S218

Status: Normal, NB-DNJ-treated

25 *Date of Birth: 4 Nov., 1997*

Gender: Female

This cat had an unremarkable developmental course before her treatment period with NB-DNJ began. Her starting dose of 1200 mg/kg/day of NB-DNJ proved to be acutely

hepatotoxic, causing a dramatic elevation in her serum levels of liver enzymes. She appeared to fully recover from the hepatotoxicity following a two week non-treatment period, so she was restarted on NB-DNJ at an eventual dosage of 100 mg/kg/day. During the remaining course of the treatment period, she exhibited some symptoms which appeared to be drug-related. Her appetite was significantly less than that of a normal cat, requiring her to be hand-fed during some intervals. Her weight gain reflected her depressed appetite, as she weighed only about 2.4 kg at the end of the treatment period. A normal female cat would be expected to weigh about 4 kg at a similar age. However, while she was exceptionally small for her age, she did not show symptoms of emaciation (e.g. muscle wasting, lethargy). Her hair colour also appeared to be affected by the NB-DNJ treatment. Her fur became markedly more beige than any other cat in the colony during the course of the treatments, even more so than the other NB-DNJ-treated animal (S222, see below). She was not subjected to neurologic assessments during the treatment period.

15

Cat number: S221

Status: NPC-affected, non-treated

Date of Birth: 4 Nov., 1997

Gender: Male

20 This cat had an unremarkable developmental course until he began exhibiting the characteristic head tremors and ataxia of feline NPC at about 10 weeks of age. Over the course of the next 20 weeks, his disease symptoms slowly worsened. By the end of the treatment period, he exhibited marked ataxia and head tremors, and required hand-feeding to maintain body weight. The following is a tabulation of his neurologic and
25 medical findings:

Animal S221 - Affected, non-treated

Week	Front leg Hopping	Rear leg hopping	Vision - menace	Ataxia	Intention Tremors	Weight (gms)*
6.5	2	2	2	none	None	544
12.5	2	2	0.5	none	Mild	994
16	2	2	1	none	Mild	1529
18.5	2	1.5	1	none	Mod	1780
20.5	2	2	2	mild	Mod	1906
22.5	2	1.5	0.5	mild	Mod	1990
24.5	2	1.5	0.5	-	Mod	2090
26.5	2	1.5	0.5	mod	Mod	2140
29	2	1.5	0.5	mild	Mod	2270
30.5	1.5	1	0.5	mod	Mod	2337
32.5	2	1	0.5	mild	Mod	2448

* measured within 10 days before corresponding neuronal assessment

Cat number: S222

Status: NPC-affected, NB-DNJ-treated

5 *Date of Birth: 4 Nov., 1997*

Gender: Female

10 This cat had an unremarkable developmental course until she began exhibiting the characteristic head tremors and ataxia of feline NPC at about 10 weeks of age. She also was noted to have bilateral luxating patellas at about the same time. As with S218, her starting dose of 1200 mg/kg/day of NB-DNJ was acutely hepatotoxic. After a no-treatment recovery period, her eventual dosage of NB-DNJ at 100 mg/kg/day was reasonably well handled. Her appetite was significantly reduced relative to both normal and NPC-affected cats, requiring her to be hand-fed often. Over the course of the next 20 weeks, her disease symptoms slowly worsened. However, on several occasions it was noted by the consulting neurologist that her symptoms were less severe than those of

15

S221. As with her affected sib, by the end of the protocol she exhibited significant ataxia and head tremors, and she required continual hand-feeding. She too had the light-coloured fur effect of NB-DNJ treatment that was noted for S218. The following is a tabulation of her neurologic and medical findings:

5

Animal S221 - Affected NB-DNJ-treated						
Week	Front leg Hopping	Rear leg Hopping	Vision - Menace	Ataxia	Intention tremors	Weight (gms)*
6.5	2	2	2	none	none	522
12.5	2	2†	1	none	mild	965
16	2	2	1	none	mild	1265
18.5	2	1.5	1.5	none	mild	1390
20.5	2	2	.5	none	mod	1453
22.5	1.5	1.5	1	none	mild	1469
24.5	2	2	1	none	mod	1495
26.5	2	1.5	1	none	mild	1525
29	2	1	1	mod	mod	1565
30.5	-	-	1	mild	mod	1590
32.5	1	1	0.5	mod	mod	1677

* measured within 10 days before corresponding neuronal assessment

† diagnosed with bilateral luxating patellas

Cat number: S161

10 *Status: Normal, NB-DNJ-treated*

Date of Birth: 17 July, 1995

Gender: Female

This cat, unrelated to the four others in the study, was included in the study to range the maximum tolerated dose of NB-DNJ in this species. Her development was unremarkable

at the time when the treatments began, save for the fact that she had a grade 3/4 heart murmur due to valvular insufficiency. She began treatment with 50 mg/kg/day of NB-DNJ on 15 March, 1998. Increasing her dose to 200 mg/kg/day brought on symptoms of lethargy, g.i. distress and increased levels of liver enzymes into her serum. Her dosage
5 was decreased to 100 mg/kg/day for the duration of the treatment period. While her appetite and overall responsiveness were decreased at this dose level, her health was sufficiently robust to maintain the treatments. Nonetheless, towards the end of the treatment period, she needed to be hand-fed to maintain her body weight. Thus, NB-DNJ treatment qualitatively delays the symptoms of neurologic degeneration typical for
10 NPC in cats.

The following sections highlight the histologic and lipid analysis findings for the study animals. As with humans, there is an increased expression of gangliosides in feline NPC neurons. Immunocytochemistry demonstrates numerous ganglioside immunoreactive
15 neurons in the cerebral cortex and cerebellum. There is a corresponding increase in neuronal ganglioside level and histology changes seen in NPC humans. Importantly, NPC cats exhibit ectopic dendrite growth similar to that seen in human children with this disease (March *et al* (1997) *Acta Neuropathol.* 94:164-172).

20 Immunocytochemical studies with anti-GM2 ganglioside antibodies were used to probe for ganglioside expression in treated vs. untreated cats in a qualitative manner. Both normal cats, regardless of treatment status, did not display GM2 immunoreactivity in pyramidal cells of the cerebral cortex, Purkinje cells, or cells within the granular layer of the cerebellum. In the NPC cat that was not treated with NB-DNJ, punctate vesicular
25 GM2 labelling was extensive and intensely labelled numerous pyramidal cells of the cerebral cortex which also displayed meganeurites. Also, Purkinje cells of the cerebral cortex and the entire granular cell layer displayed extensive GM2 labelling. In the NPC cat treated with NB-DNJ, GM2 labelling was observed in the cerebral cortex, but was

qualitatively less severe compared to the untreated cat. In the cerebellum, the granular cell layer was largely devoid of GM2 immunoreactivity, suggesting that ganglioside storage had been qualitatively diminished relative to that seen in the untreated NPC cat. Purkinje cells also demonstrated qualitatively less GM2 labelling. Thus, NB-DNJ treatment qualitatively decreases the accumulation of glucosylceramide-containing glycolipids (e.g. GM2) typical for NPC in cats.

Example 2 – Inhibition of clinical and pathological symptoms in a mouse model of NPC

Colonies of mutant mice expressing the NPC phenotype have been described (Pentchev *et al.*, 1984, Miyawaki *et al.*, 1986; Kitagawa, 1987), and has been validated by a number of criteria as an authentic model of the disease (Akaboshi *et al.*, 1997). NPC mice display clinical signs of the disease around 6-8 weeks of age with mild intention tremor and ataxia. By 9 weeks, the mice exhibit severe ataxia, tremors and weight loss. Death results by 10-12 weeks.

The brains of NPC mice are grossly normal. However, microscopic examination reveals swollen somata, meganeurite formation and enlarged axon hillock regions of cortical pyramidal neurons. Meganeurites and neuritic tufts appear in amygdala neuron. White matter and Purkinje cells display axonal spheroids. Anti-ganglioside antibody staining shows increased GM2 levels primarily in laminae II/III and V pyramidal neurons, and astrocytes in layer I. GD2 levels are elevated in pyramidal neurons throughout the cerebral cortex. Moderate increases are also seen for level of GM3 in layer VI, and GM1 in pyramidal neurons. There is no corresponding change in CD3 or asialo-GM2 levels in NPC mouse brains.

Breeding pairs of mice heterozygous for the mutation causing NPC were used to produce offspring that are NPC^{-/-} homozygotes. These animals, along with their normal wildtype

littermates, were used in the following NB-DNJ drug study. Where indicated, NB-DNJ was administered daily by mixing with ground mouse chow. Mice were PCR genotyped 2-3 weeks of age to determine their genetic background.

- 5 Ten NPC^{-/-} mice, with ages ranging from 3-7 weeks, were entered into a treatment study. Seven were treated with 1200 mg/kg/day and six were untreated. Regardless of treatment, NPC mice between the ages of 0-5 weeks did not display any features of the NPC phenotype. However, by 8 weeks of age, 5 out of 6 untreated NPC^{-/-} mice displayed the clinical phenotype of their disease (intention tremor, ataxia), while none of
10 the NB-DNJ displayed any symptoms of neurologic effects. All six of the untreated mice showed severe neurologic impairment by 9 weeks of age, whereas only 4 of 7 NB-DNJ treated mice displayed any degree of symptoms. By 10 weeks of age, all six untreated NPC^{-/-} mice died or were sacrificed according to veterinary animal care requirements. In contrast, 4 of 7 NPC^{-/-} mice treated with NB-DNJ lived into their twelfth week. Three
15 of these four surviving mice displayed some degree of NPC-induced neural degeneration, while one appeared normal. In this experiment, untreated NPC^{-/-} mice survived 65 ± 1 days (average \pm SE; n = 6), while NPC^{-/-} mice treated with NB-DNJ at 1200 mg/kg/day survived 88 ± 4 days (n = 7). Thus, NB-DNJ treatment increase longevity in NPC mice by 26% in this study, as well as qualitatively delaying the symptoms of neurologic
20 degeneration typical for NPC in mice.

CLAIMS:

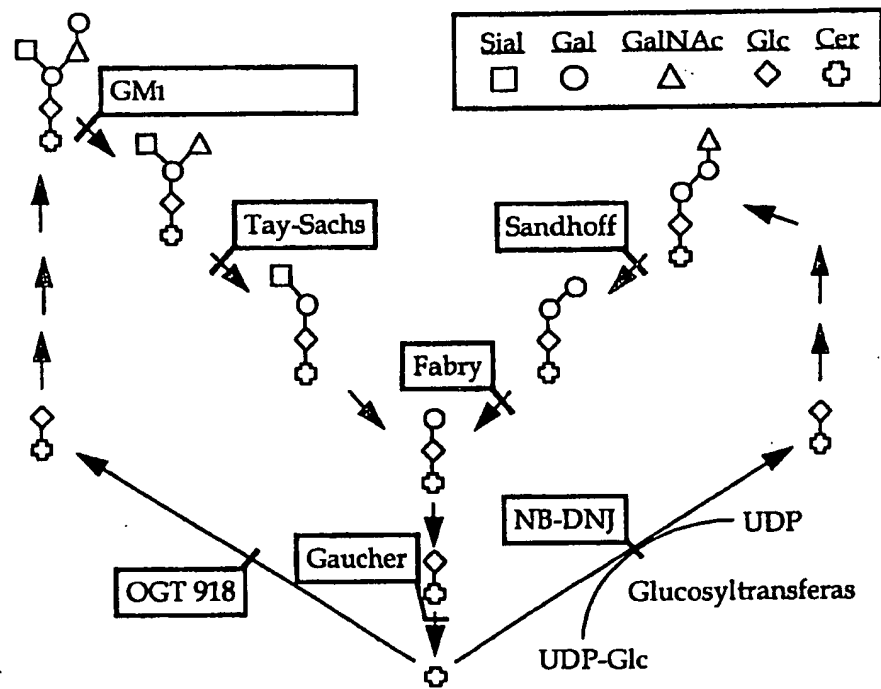
1. The use of an inhibitor of glucosylceramide synthesis in the manufacture of a medicament for use in the treatment of Niemann-Pick type C disease.
5
2. The use of an inhibitor of glucosylceramide synthesis in the treatment of Alzheimer's disease.
3. The use of an inhibitor of glucosylceramide synthesis in the treatment of epilepsy.
10
4. The use as claimed in claim 1, 2 or 3, wherein the inhibitor is one or more imino sugar-structured inhibitors of glucosylceramide synthesis.
5. The use as claimed in claim 4, wherein the inhibitor comprises one or more of N-butyldeoxynojirimycin, N-butyldeoxygalactonojirimycin and N-nonyldeoxynojirimycin.
15
6. The use as claimed in any preceding claim, wherein the inhibitor comprises 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol or a structurally related analogue thereof.
20
7. The use as claimed in any preceding claim, wherein the inhibitor comprises one or more of a nucleic acid coding for a protein or peptide capable of inhibiting glucosylceramide synthesis, and an antisense sequence or catalytic RNA capable of interfering with the expression of enzymes responsible for glucosylceramide synthesis.
25
8. The use of N-butyldeoxynojirimycin in the manufacture of a medicament for use in the treatment of Niemann-Pick type C disease.

9. The use of an agent capable of increasing the rate of neuronal glycolipid degradation in the manufacture of a medicament for use in the treatment of Niemann-Pick type C disease.
- 5 10. The use of an agent capable of increasing the rate of neuronal glycolipid degradation in the manufacture of a medicament for use in the treatment of Alzheimer's disease.
- 10 11. The use of an agent capable of increasing the rate of neuronal glycolipid degradation in the manufacture of a medicament for use in the treatment of epilepsy.
- 15 12. The use as claimed in claim 9, 10 or 11, wherein the agent comprises one or more of an enzyme which degrades neuronal glycolipids, a molecule which increases the activity of such an enzyme, and a nucleic acid sequence (DNA or RNA) which codes for such an enzyme.
- 20 13. The use of an inhibitor of glucosylceramide synthesis in the production of a medicament for the treatment of a condition treatable by the administration of a ganglioside.
14. The use as claimed in claim 13, wherein the condition is treatable by the administration of GM1 ganglioside.
- 25 15. The use as claimed in claim 13 or claim 14, wherein the condition is Parkinson's disease, stroke or a spinal cord injury.
16. The use as claimed in claim 13, 14 or 15, wherein the medicament further comprises a ganglioside.

17. The use as claimed in claim 16, wherein the ganglioside is GM1 ganglioside.
18. A product comprising an inhibitor of glucosylceramide synthesis and a
5 ganglioside as a combined preparation for simultaneous, sequential or separate use in the
treatment of a condition treatable by the administration of a ganglioside.
19. A product as claimed in claim 18, wherein the ganglioside is GM1 ganglioside.

1/1

FIGURE 1



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/01563

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K31/445 A61K31/5375 A61P3/00 A61P25/00 A61P25/08
A61P25/16 A61P25/28 A61P9/10 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPQ-Internal, CHEM ABS Data, MEDLINE, EMBASE, WPI Data, PAJ, BIOSIS, CANCERLIT, AIDSLINE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	WO 98 02161 A (VIANELLO PAOLA ;KOOMEN GERRIT JAN (NL); AERTS JOHANNES MARIA FRANC) 22 January 1998 (1998-01-22) abstract page 1, line 1 - line 5 page 2, line 32 -page 3, line 3 page 4, line 15 - line 17 page 8, line 26 - line 36 page 9, line 24 - line 26 page 14, line 6 - line 17 page 14, line 31 - line 36 page 23, line 5 - line 18 claims 12-15 --- -/--	1,4 5,8,9

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *8* document member of the same patent family

Date of the actual completion of the international search

15 September 2000

Date of mailing of the international search report

06/10/2000

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Authorized officer

Cielen, E

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 00/01563

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>PLATT F M ET AL: "NEW THERAPEUTIC PROSPECTS FOR THE GLYCOSPHINGOLIPID LYSOSOMAL STORAGE DISEASES" BIOCHEMICAL PHARMACOLOGY, GB, PERGAMON, OXFORD, vol. 56, no. 4, 1998, pages 421-430, XP000886851 ISSN: 0006-2952 abstract figures 1,2 page 423, column 2, paragraph 2 page 424, column 1, paragraph 3 -column 2, paragraph 2 page 425, column 2, paragraph 2 page 426, column 1, paragraph 1 -page 427, column 1, paragraph 3 page 429, column 1, paragraph 2</p>	1,4-6,8
Y	<p>US 5 798 366 A (BUTTERS TERRY D ET AL) 25 August 1998 (1998-08-25) abstract column 1, line 26 - line 32 column 1, line 65 -column 2, line 59 column 7, line 12 - line 42</p>	1,4-6,8
Y	<p>PLATT F M ET AL: "PREVENTION OF LYSOSOMAL STORAGE IN TAY-SACHS MICE TREATED WITH N-BUTYLDEOXYNOJIRIMYCIN" SCIENCE, US, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 276, no. 5311, 18 April 1997 (1997-04-18), pages 428-431, XP002065772 ISSN: 0036-8075 abstract page 429, column 1, paragraph 2 - paragraph 3 page 430, column 3, paragraph 3 -page 431, column 1, paragraph 1</p> <p style="text-align: center;">-/--</p>	1,4,5,8

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 00/01563

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>PLATT F M ET AL: "N-BUTYLDEOXYGALACTONOJIRIMYCIN INHIBITS GLYCOLIPID BIOSYNTHESIS BUT DOES NOT AFFECT N-LINKED OLIGOSACCHARIDE PROCESSING" JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 269, no. 43, 28 October 1994 (1994-10-28), pages 27108-27114, XP002065777 ISSN: 0021-9258 abstract page 27108, column 1, paragraph 2 -column 2, paragraph 3 page 27110, column 2, paragraph 2 page 27111, column 1, paragraph 3 -column 2, paragraph 1 page 27114, column 1, paragraph 4 -column 2, paragraph 1</p> <p>---</p>	1,4,5,8
Y	<p>M. H. BEERS, R. BERKOW: "The Merck Manual of Diagnosis and Therapy Seventeenth Edition" 1999, MERCK RESEARCH LABORATORIES, NEW YORK XP002147345 page 212 -page 216</p> <p>---</p>	1,4-6,8
X	<p>RÖSNER HARALD: "Significance of gangliosides in neuronal differentiation of neuroblastoma cells and neurite growth in tissue culture." ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol. 845, 16 June 1998 (1998-06-16), pages 200-214, XP000937684 abstract page 200, paragraph 2 page 201, paragraph 3 page 204, paragraph 3 table 1 page 206, paragraph 2 page 209, paragraph 1 - paragraph 2 page 210, paragraph 1 page 211, paragraph 3 page 212, paragraph 3 -page 213, paragraph 1</p> <p>---</p> <p style="text-align: center;">-/--</p>	13-19

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/01563

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	MEUILLET E J ET AL: "Modulation of EGF receptor activity by changes in the GM3 content in a human epidermoid carcinoma cell line, A431." EXPERIMENTAL CELL RESEARCH, (2000 APR 10) 256 (1) 74-82. , XP000937516	13, 16, 18
P, A	abstract page 75, column 1, paragraph 1 page 78, column 2, paragraph 1 -page 79, column 1, paragraph 1 page 79, column 2, paragraph 1 ----	14, 17, 19
E	WO 00 33843 A (UNIV OXFORD ;BUTTERS TERRY D (GB); DWEK RAYMOND A (GB); PLATT FRAN) 15 June 2000 (2000-06-15) abstract page 3, paragraph 1 - paragraph 2 page 5, paragraph 3 page 7, paragraphs 2,4 claims 1-3 -----	1, 4, 5, 8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 00 01563

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-4,7,13-19 relate to a compounds, compositions and their therapeutic uses defined by reference to a desirable characteristic or property, namely "an inhibitor of glucosylceramide synthesis". Moreover, claim 7, claims 9-12 relate to compounds and their therapeutic uses defined by reference to the desirable characteristics or properties "a nucleic acid coding for a protein or peptide capable of inhibiting glucosylceramide synthesis", "an antisense sequence or catalytic RNA capable of interfering with the expression of enzymes responsible for glucosylceramide synthesis", "an agent capable of increasing the rate of neuronal glycolipid degradation", "an enzyme which degrades neuronal glycolipids", "a molecule which increases the activity of such an enzyme", "a nucleic acid sequence (DNA or RNA) which codes for such an enzyme".

The claims cover all compounds, compositions and their therapeutic uses having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds, compositions and their therapeutic uses. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds, compositions and their therapeutic uses by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Moreover, present claims 13-14,16-19 relate to a therapeutic application defined as "a treatment of a condition treatable by administration of a ganglioside". The definition is not a clear and unequivocal description of a therapeutic application. The expression "a structurally related analogue" is vague and indeterminate (claim 6). Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds for which pharmaceutical data are provided in the examples and the compounds specifically mentioned in claims 5, 6, 8 and the diseases specifically mentioned in claims 1-3, 8-11 and 15, with due regard to the general idea underlying the application.

Claims searched partially: 1-19.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 00/01563

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9802161 A	22-01-1998	AU 3464797 A EP 0912179 A	09-02-1998 06-05-1999
US 5798366 A	25-08-1998	US 5656641 A US 5786368 A US 5580884 A US 5399567 A AU 5813898 A EP 1007043 A WO 9830219 A US 5786369 A US 5801185 A AT 148456 T AU 6783294 A CA 2159988 A DE 69401658 D DE 69401658 T DK 698012 T EP 0698012 A ES 2097653 T GR 3022554 T JP 8510244 T WO 9426714 A US 5472969 A US 5525616 A	12-08-1997 28-07-1998 03-12-1996 21-03-1995 03-08-1998 14-06-2000 16-07-1998 28-07-1998 01-09-1998 15-02-1997 12-12-1994 24-11-1994 13-03-1997 12-06-1997 17-02-1997 28-02-1996 01-04-1997 31-05-1997 29-10-1996 24-11-1994 05-12-1995 11-06-1996
WO 0033843 A	15-06-2000	NONE	

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



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(54) Title: LONG CHAIN N-ALKYL COMPOUNDS AND OXA-DERIVATIVES THEREOF

(57) Abstract: Long chain N-alkyl amino and imino compounds, oxa-substituted derivatives thereof, and pharmaceutical compositions including such compounds are described. The long chain N-alkyl group is a C₈-C₁₆ alkyl group. The long chain N-alkyl compounds and oxa-substituted derivatives thereof can be used in the treatment of viral infections, in particular hepatitis B virus or hepatitis C virus, in a cell or an individual. For example, the long chain N-alkyl compounds or oxa-substituted derivatives thereof can be derived from piperidines, pyrrolidines, phenylamines, pyridines, pyrroles, or amino acids.

LONG CHAIN N-ALKYL COMPOUNDS AND OXA-DERIVATIVES THEREOF

FIELD OF THE INVENTION

This invention relates to long chain N-alkyl amino and imino compounds and oxa-
5 derivatives thereof for treating pestivirus and flavivirus infections of animals and humans.

BACKGROUND OF THE INVENTION

HCV is an RNA virus belonging to the *Flaviviridae* family. Individual isolates consist of closely related, yet heterologous populations of viral genomes. This genetic
10 diversity enables the virus to escape the host's immune system, leading to a high rate of chronic infection. The flavivirus group to which HCV belongs is known to include the causative agents of numerous human diseases transmitted by arthropod vectors. Human diseases caused by flaviviruses include various hemorrhagic fevers, hepatitis, and encephalitis. Viruses known to cause these diseases in humans have been identified and
15 include, for example, yellow fever virus, dengue viruses 1-4, Japanese encephalitis virus, Murray Valley encephalitis virus, Rocio virus, West Nile fever virus, St. Louis encephalitis virus, tick-borne encephalitis virus, Louping ill virus, Powassan virus, Omsk hemorrhagic fever virus, and Kyasanur forest disease virus. A critical need therefore also exists for treating animals, as well as humans, infected with at least one virus, such as a flavivirus
20 and/or pestivirus.

More than 40 million people worldwide are chronically infected with the hepatitis C virus (HCV), and this represents one of the most serious threats to the public health of developed nations (Hoofnagle et al., *New Engl. J. Med.* 336:347-356, 1997). Hepatitis C infection is the cause of more than 10,000 deaths annually in the United States (*Washington*
25 *Post*, November 11, 1997, at A2), a number that is expected to triple in the next twenty years in the absence of effective intervention. Chronic HCV also increases the risk of liver cancer. There are more than 40 million people worldwide who are chronically infected with HCV, representing one of the most serious threats to the public health of developed nations (Hoofnagle et al., *ibid.*). Persistent infection develops in as many as 85% of HCV patients
30 and in at least 20% of these patients the chronic infection leads to cirrhosis within twenty years of onset of infection. With an estimated 3.9 million North Americans chronically infected, complications from hepatitis C infection are now the leading reasons for liver transplantation in the United States.

Another causative agent of acute and chronic liver disease including liver fibrosis, cirrhosis, inflammatory liver disease, and hepatic cancer is hepatitis B virus (HBV) (Joklik, *Virology*, 3rd Ed., Appleton & Lange, Norwalk, Connecticut, 1988). Although effective vaccines are available, there are still more than 300 million people worldwide, i.e., 5% of the world's population, chronically infected with the virus (Locamini et al., *Antiviral Chemistry & Chemotherapy* 7:53-64, 1996). Such vaccines have no therapeutic value for those already infected with the virus. In Europe and North America, between 0.1% to 1% of the population is infected. Estimates are that 15% to 20% of individuals who acquire the infection develop cirrhosis or another chronic disability from HBV infection. Once liver cirrhosis is established, morbidity and mortality are substantial, with about a 5-year patient survival period (Blume et al., *Advanced Drug Delivery Reviews* 17:321-331, 1995). It is therefore necessary and of high priority to find improved and effective anti-HBV anti-hepatitis therapies (Locamini et al., *ibid.*).

Therapeutic interventions which are effective for treatment of HCV infection are limited in number and effectiveness. Standard treatment for HCV infection includes administration of interferon-alpha. However, interferon-alpha is of limited use in about 20% of the HCV-infected population (Hoofnagle et al., *ibid.*) and treatment with this compound results in long-term improvement in only 5% of patients. Furthermore, the complications and limitations of interferon-alpha seriously limit the applicability of the treatment. An experimental treatment comprising administration of interferon-alpha and ribavirin (1- β -D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide) resulted in long-term improvement in only half of the patients suffering a relapse of HCV infection (*Washington Post*, November 11, 1997, at A2). Clearly, the disappointing results with interferon must prompt a search for more effective and less toxic therapeutics. Thus, a critical need remains for a therapeutic intervention that effectively treats HCV infection or supplements those otherwise available.

In addition to those people chronically infected with HCV, there are more than 350 million people chronically infected with hepatitis B virus (HBV). More than 150 million of these people are likely to die from liver disease in the absence of intervention. As many as 20 million HBV carriers reside in developed nations, as do most HCV carriers. A large number of individuals who are infected with HCV are also infected with HBV. The therapy for combined HBV/HCV infection is particularly challenging because the HBV and HCV viruses differ from one another in therapeutically significant ways. HBV is a hepadnavirus, while HCV is a pestivirus. HBV is a DNA-containing virus, the genome of which is

replicated in the nucleus of the infected cell using a combination of a DNA-dependent RNA polymerase and an RNA-dependent DNA polymerase (i.e., a reverse transcriptase). HCV is an RNA-containing virus, the genome of which is replicated in the cytoplasm of the infected cell using one or more types of RNA-dependent RNA polymerases. Despite the frequent
5 concurrence of HBV infection and HCV infection, a number of compounds known to be effective for treating HBV infection are not effective against HCV. For example, lamivudine (the nucleoside analog 3TC) is useful for treating HBV infection, but is not useful for treating HCV infection. The difference in the susceptibility of HBV and HCV to antiviral agents no doubt relates to their genetically based replicative differences. There remains a particularly
10 critical need for a therapeutic intervention that effectively treats both HBV and HCV infection.

Other hepatitis viruses significant as agents of human disease include hepatitis A, hepatitis Delta, hepatitis E, hepatitis F, and hepatitis G (Coates et al., *Exp. Opin. Ther. Patents* 5:747-756, 1995). In addition, there are animal hepatitis viruses that are species
15 specific. These include, for example, those infecting ducks, woodchucks, and mice. The availability of animal models allows the preclinical testing of antiviral compounds for each class of virus. Furthermore, animal viruses can cause significant losses to the livestock industry (Sullivan et al., *Virus Res.* 38:231-239, 1995). Such animal viruses include pestiviruses and flaviviruses such as bovine viral diarrhea virus (BVDV), classical swine
20 fever virus, border disease virus, and hog cholera virus.

SUMMARY OF THE INVENTION

In general, the invention features long chain N-alkyl amino and imino compounds and oxa-substituted derivatives thereof and includes pharmaceutical compositions containing an
25 effective amount of such compounds. The long chain N-alkyl group is a C₈-C₁₆ alkyl group. The long chain N-alkyl compounds and oxa-substituted derivatives thereof can be used in the treatment of viral infections in a cell or an individual. In an individual, the infection may result in chronic or acute disease and treatment of same may reduce the severity of infection (e.g., production of virus) or disease symptoms. The long chain N-alkyl compounds may or
30 may not inhibit glycosidase activity or glycolipid synthesis at a detectable level; preferred are compounds that do not inhibit α -glucosidase activity at a detectable level but still are effective in treating infection. For example, the long chain N-alkyl compounds and oxa-substituted derivatives can be derived from a piperidine, a pyrrolidine, a phenylamine, a

pyridine, a pyrrole, or an amino acid.

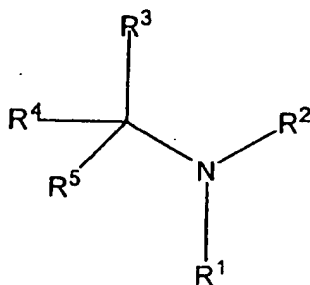
In one aspect, the invention features a nitrogen-containing virus-inhibiting compound including an N-C₈-C₁₆ alkyl group. Preferably, the compound includes an N-C₈-C₁₀ alkyl group (e.g., N-nonyl or N-decyl group) or an N-C₈-C₁₀ oxa-alkyl group such as an N-(CH₂)₆O(CH₂)_nCH₃ group or N-(CH₂)₂O(CH₂)_{n+4}CH₃ group for n = 1, 2 or 3. The nitrogen-containing virus-inhibiting compound can have an inhibitory concentration (IC₅₀) of about 20 μM or less, preferably about 10 μM or less, and more preferably about 5 μM or less, for the inhibition of one or more pestiviruses or a flaviviruses in an assay (e.g., plaque formation, yield). In particular, a compound effective against both a pestivirus and a flavivirus (e.g., HBV and BVDV) is preferred.

In another aspect, the invention features a method of inhibiting morphogenesis of a virus. The method includes administering an effective amount of the nitrogen-containing virus-inhibiting compound, or a pharmaceutically acceptable salt thereof, to a cell or an individual infected with the virus. The cell can be a mammalian cell or a human cell.

In yet another aspect, the invention features a method of treating an individual infected with a virus. The method includes administering an effective amount of the nitrogen-containing virus-inhibiting compound, or a pharmaceutically acceptable salt thereof, to an individual infected with a virus. The treatment can reduce, abate, or diminish the virus infection in the animal or human. The animal can be a bird or mammal (e.g., pig, cow, mice). The nitrogen-containing virus-inhibiting compound can be administered orally.

In another aspect, the invention features a method of manufacturing a pharmaceutical composition comprising combining at least one nitrogen-containing virus-inhibiting compound including an N-C₈-C₁₆ alkyl group or an oxa-substituted derivative thereof with a pharmaceutically acceptable carrier.

The compound can have the formula:



in which R¹ is a C₈-C₁₆ alkyl; and can also contain 1 to 5, preferably 1 to 3, and more preferably 1 to 2 oxygen atoms (i.e., oxa-substituted derivatives). Preferred oxa-substituted

derivatives are 3-oxanonyl, 3-oxadecyl, 7-oxanonyl and 7-oxadecyl.

R^2 is hydrogen, R^3 is carboxy, or a C_1 - C_4 alkoxycarbonyl, or R^2 and R^3 , together are

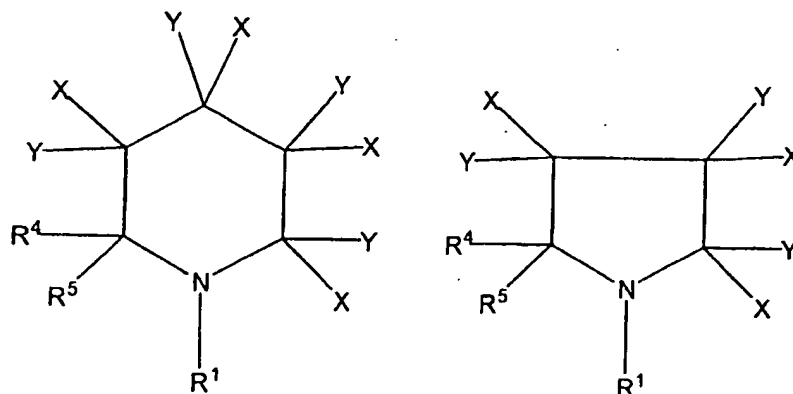
5 $\begin{array}{c} X \quad Y \\ \backslash \quad / \\ -(C)_n- \text{ or } -(CXY)_n- \end{array}$, wherein n is 3 or 4, each X , independently, is hydrogen, hydroxy, amino, carboxy, a C_1 - C_4 alkylcarboxy, a C_1 - C_4 alkyl, a C_1 - C_4 alkoxy, a C_1 - C_4 hydroxyalkyl, a C_1 - C_6 acyloxy, or an aroyloxy, and each Y , independently, is hydrogen, hydroxy, amino, carboxy, a C_1 - C_4 alkylcarboxy, a C_1 - C_4 alkyl, a C_1 - C_4 alkoxy, a C_1 - C_4 hydroxyalkyl, a C_1 - C_6 acyloxy, an aroyloxy, or deleted (i.e., not present);

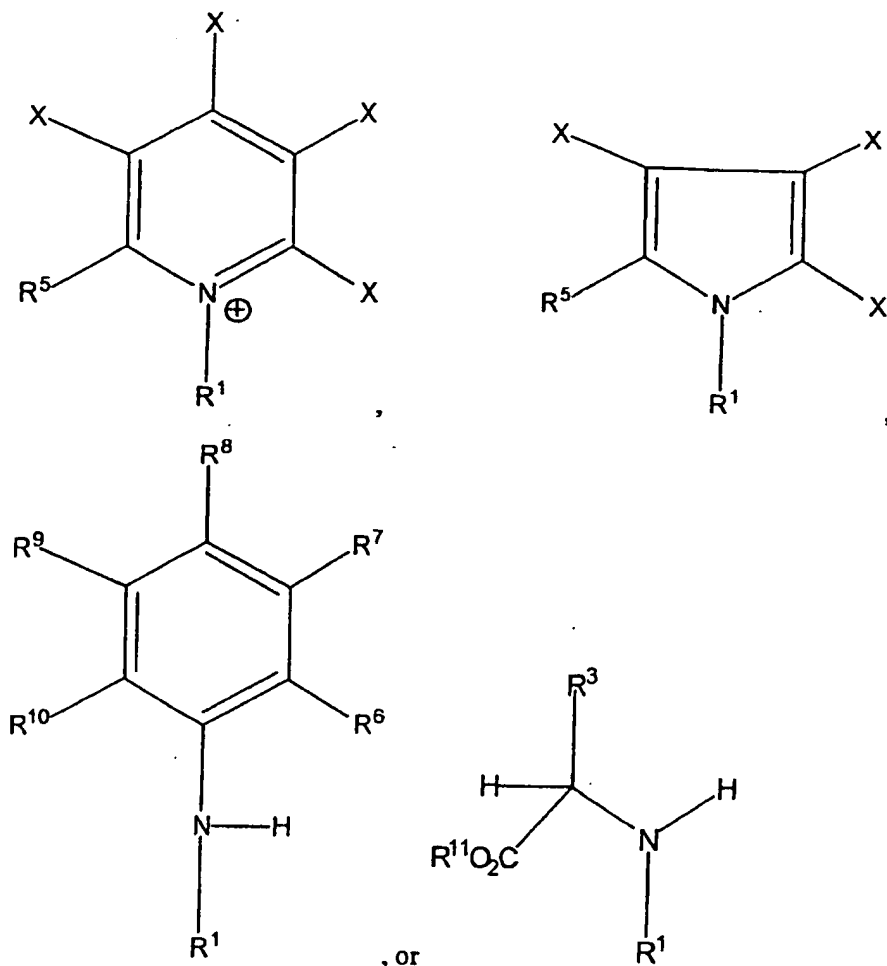
10 R^4 is hydrogen or deleted (i.e., not present); and

R^5 is hydrogen, hydroxy, amino, a substituted amino, carboxy, an alkoxycarbonyl, an aminocarbonyl, an alkyl, an aryl, an aralkyl, an alkoxy, a hydroxyalkyl, an acyloxy, or an aroyloxy, or R^3 and R^5 , together, form a phenyl and R^4 is deleted (i.e., not present). When R^2 and R^3 , together, are $-(CXY)_n-$ and R^4 is deleted (i.e., not present), all Y are deleted (i.e., not present). The compound can be a physiologically acceptable salt or solvate of the compound.

In certain embodiments, R^1 is a C_8 - C_{10} alkyl (e.g., C_9 alkyl) and R^2 can be hydrogen, R^3 can be carboxy, or a C_1 - C_4 alkoxycarbonyl, R^4 can be hydrogen, and R^5 can be hydrogen, hydroxy, amino, a substituted amino, carboxy, an alkoxycarbonyl, an aminocarbonyl, an alkyl, an aryl, an aralkyl, an alkoxy, a hydroxyalkyl, an acyloxy, or an aroyloxy. In certain preferred embodiments, R^3 is carboxy. In other preferred embodiments, R^3 and R^5 , together, form a phenyl and R^4 is deleted (i.e., not present). In yet other preferred embodiments, R^2 and R^3 , together, are $-(CXY)_n-$.

In certain embodiments, the compound has the formula:





Each of R^6 - R^{10} , independently, is hydrogen, hydroxy, amino, carboxy, a C_1 - C_4 alkylcarboxy, a C_1 - C_4 alkyl, a C_1 - C_4 alkoxy, a C_1 - C_4 hydroxyalkyl, a C_1 - C_6 acyloxy, or an aroyloxy, and R^{11} is hydrogen, or a C_1 - C_4 alkyl.

The nitrogen-containing virus inhibiting compound can be N-alkylated piperidines, N-oxa-alkylated piperidines, N-alkylated pyrrolidines, N-oxa-alkylated pyrrolidines, N-alkylated phenylamines, N-oxa-alkylated phenylamines, N-alkylated pyridines, N-oxa-alkylated pyridines, N-alkylated pyrroles, N-oxa-alkylated pyrroles, N-alkylated amino acids, or N-oxa-alkylated amino acids. In certain embodiments, the N-alkylated piperidine, N-oxa-alkylated piperidine, N-alkylated pyrrolidine, or N-oxa-alkylated pyrrolidine compound can be an imino sugar. For example, preferred nitrogen-containing virus-inhibiting compounds are N-nonyl-1,5-dideoxy-1,5-imino-D-galactitol (N-nonyl-deoxygalactonojirimycin or N-nonyl DGJ), N-(7-oxa-nonyl)-1,5-dideoxy-1,5-imino-D-galactitol (N-7-oxa-nonyl DGJ), N-nonyl-1,5,6-trideoxy-1,5-imino-D-galactitol (N-nonyl MeDGJ), N-(7-oxa-nonyl)-1,5,6-

trideoxy-1,5-imino-D-galactitol (N-7-oxa-nonyl MeDGJ), N-nonyl altrostatin, N-nonyl-2*R*,5*R*-dihydroxymethyl-3*R*,4*R*-dihydroxypyrrolidine (N-nonyl DMDP), N-nonyl-deoxynojirimycin (N-nonyl DNJ), N-nonyl-2-aminobenzamide (2ABC9), or a derivative, an enantiomer or a stereoisomer thereof. The structures of unsubstituted compounds are shown
5 in Figure 1.

In certain embodiments, the virus can be a flavivirus or a pestivirus. Infections by flaviviruses include, but are not limited to, those caused by a yellow fever virus, a dengue virus (e.g., dengue viruses 1-4), a Japanese encephalitis virus, a Murray Valley encephalitis virus, a Rocio virus, a West Nile fever virus, a St. Louis encephalitis virus, a tick-borne
10 encephalitis virus, a Louping ill virus, a Powassan virus, an Omsk hemorrhagic fever virus, and a Kyasanur forest disease virus. Infections by pestiviruses include, but are not limited to, those caused by hepatitis C virus (HCV), rubella virus, a bovine viral diarrhea virus (BVDV), a classical swine fever virus, a border disease virus, or a hog cholera virus.

According to yet another aspect, the invention features a prophylactic method for
15 protecting a mammal infected by a virus from developing hepatitis or a hepatocellular cancer that is among the sequelae of infection by the virus, including administering to the virus infected cell of the animal an effective anti-viral amount of the nitrogen-containing virus-inhibiting compound.

20 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts chemical structures for compounds which were used in this study.

Figure 2 depicts the percent of BVDV plaques produced by an infected cell culture in the presence of various concentrations of compounds: N-butyl DGJ (◆), N-nonyl DGJ (■), N-nonyl MeDGJ (▲), or N-nonyl DNJ(×).

25 Figures 3 depicts the IC₅₀ of various alkyl lengths of N-alkylated compounds and Figure 5 depicts the IC₅₀ of N-nonyl compounds.

Figure 4 depicts the percent of BVDV plaques produced by an infected cell culture in the presence of various concentrations of N-nonyl DGJ (▲) or N-decyl DGJ (×).

Figure 6 depicts the percent of BVDV plaques produced by an infected cell culture in
30 the presence of various concentrations of N-nonyl compounds: 2ABC9 (◆), nonylamine (■), N-nonyl-altrostatin (△), N-nonyl-DGJ (×), N-nonyl-MeDGJ (✱), N-nonyl-DNJ (●), or N-nonyl-DMDP (+).

Figure 7 depicts the percent of BVDV plaques produced by an infected cell culture in

the presence of various concentrations of N-7-oxa-nonyl MeDGJ.

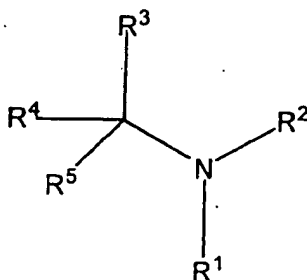
Figure 8 depicts the increasing uptake of ^3H -labeled inhibitors in HepG2 cells in the following order: N-butyl-DNJ (◆), N-hexyl-DNJ (■), N-octyl-DNJ (▲), N-nonyl-DNJ (×), N-decyl-DNJ (⋈), N-dodecyl-DNJ (●), N-hexadecan-DNJ (+), or N-octadecan-DNJ (—).

5

DESCRIPTION OF THE INVENTION

The nitrogen-containing virus-inhibiting compound includes an N-C₈-C₁₆ alkyl group, such as an N-C₈-C₁₀ alkyl group, particularly a nonyl or decyl group, or an oxa-substituted derivative thereof. The nitrogen-containing virus-inhibiting compound can be an N-alkylated
 10 piperidine, N-oxa-alkylated piperidine, N-alkylated pyrrolidine, N-oxa-alkylated pyrrolidine, N-alkylated phenylamine, N-oxa-alkylated phenylamine, N-alkylated pyridine, N-oxa-alkylated pyridine, N-alkylated pyrrole, N-oxa-alkylated pyrrole, N-alkylated amino acid, or N-oxa-alkylated amino acid such as N-nonyl DGJ, N-oxa-nonyl DGJ, N-nonyl MeDGJ, N-oxa-nonyl MeDGJ, N-nonyl altrostatin, N-nonyl DMDP, N-oxa-nonyl DMDP, N-nonyl-2-
 15 aminobenzamide, or N-oxa-nonyl-2-aminobenzamide.

The compound can have the formula:



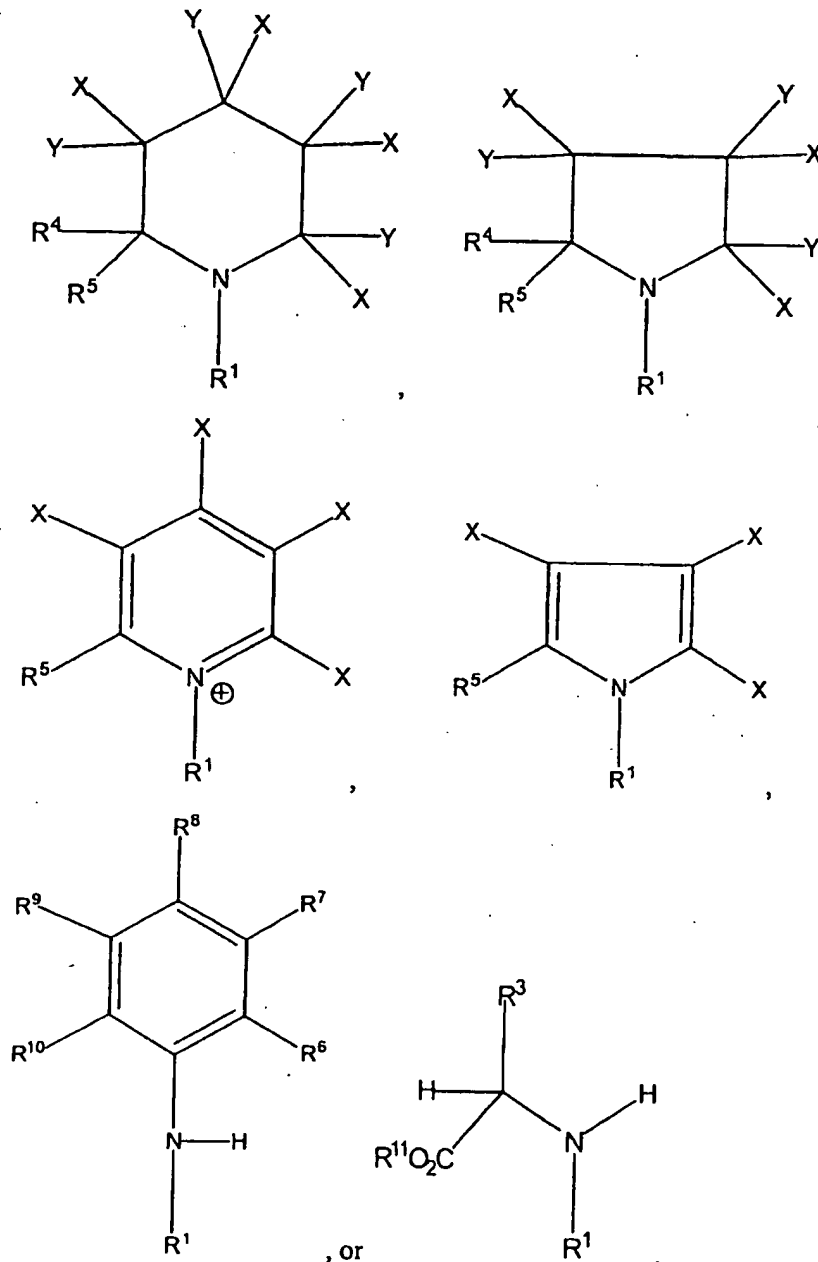
in which R¹ is a C₈-C₁₆ alkyl, R² is hydrogen, R³ is carboxy, or a C₁-C₄ alkoxy-carbonyl, R⁴ is hydrogen, and R⁵ is hydrogen, hydroxy, amino, a substituted amino, carboxy, an
 20 alkoxy-carbonyl, an aminocarbonyl, an alkyl, an aryl, an aralkyl, an alkoxy, a hydroxyalkyl, an acyloxy, or an aroyloxy. Alternatively, R¹ is a C₈-C₁₆ alkyl, R² is hydrogen, R³ and R⁵, together, form a phenyl, which can be substituted or unsubstituted, and R⁴ is deleted (i.e., not present). In another alternative, R¹ is a C₈-C₁₆ alkyl, R⁴ is hydrogen or deleted (i.e., not present), R⁵ is hydrogen, hydroxy, amino, a substituted amino, carboxy, an alkoxy-carbonyl,
 25 an aminocarbonyl, an alkyl, an aryl, an aralkyl, an alkoxy, a hydroxyalkyl, an acyloxy, or an



aroyloxy, and R² and R³, together, are -(C)_n- or -(CXY)_n-, wherein n is 3 or 4, each X, independently, is hydrogen, hydroxy, amino, carboxy, a C₁-C₄ alkylcarboxy, a C₁-C₄ alkyl, a

- C₁-C₄ alkoxy, a C₁-C₄ hydroxyalkyl, a C₁-C₆ acyloxy, or an aroyloxy, and each Y, independently, is hydrogen, hydroxy, amino, carboxy, a C₁-C₄ alkylcarboxy, a C₁-C₄ alkyl, a C₁-C₄ alkoxy, a C₁-C₄ hydroxyalkyl, a C₁-C₆ acyloxy, an aroyloxy, or deleted. When R² and R³, together, are -(CXY)_n- and R⁴ is deleted, all Y are deleted. The compound can be a
- 5 physiologically acceptable salt or solvate of the compound.

In certain embodiments, the compound has the formula:



Each of R⁶-R¹⁰, independently, is hydrogen, hydroxy, amino, carboxy, a C₁-C₄

alkylcarboxy, a C₁-C₄ alkyl, a C₁-C₄ alkoxy, a C₁-C₄ hydroxyalkyl, a C₁-C₆ acyloxy, or an aroyloxy, and R¹¹ is hydrogen, or a C₁-C₄ alkyl.

As used herein, the groups have the following characteristics, unless the number of carbon atoms is specified otherwise. Alkyl groups have from 1 to 16 carbon atoms and are linear or branched, substituted or unsubstituted. Alkoxy groups have from 1 to 16 carbon atoms, and are linear or branched, substituted or unsubstituted. Alkoxycarbonyl groups are ester groups having from 2 to 16 carbon atoms. Alkenyloxy groups have from 2 to 16 carbon atoms, from 1 to 6 double bonds, and are linear or branched, substituted or unsubstituted. Alkynyloxy groups have from 2 to 16 carbon atoms, from 1 to 3 triple bonds, and are linear or branched, substituted or unsubstituted. Aryl groups have from 6 to 14 carbon atoms (e.g., phenyl groups) and are substituted or unsubstituted. Aralkyloxy (e.g., benzyloxy) and aroyloxy (e.g., benzoyloxy) groups have from 7 to 15 carbon atoms and are substituted or unsubstituted. Amino groups can be primary, secondary, tertiary, or quaternary amino groups (i.e., substituted amino groups). Aminocarbonyl groups are amido groups (e.g., substituted amido groups) having from 1 to 32 carbon atoms. Substituted groups can include a substituent selected from the group consisting of halogen, hydroxy, C₁₋₁₀ alkyl, C₂₋₁₀ alkenyl, C₁₋₁₀ acyl, or C₁₋₁₀ alkoxy.

The N-alkylated amino acid can be an N-alkylated naturally occurring amino acid, such as an N-alkylated α -amino acid. A naturally occurring amino acid is one of the 20 common α -amino acids (Gly, Ala, Val, Leu, Ile, Ser, Thr, Asp, Asn, Lys, Glu, Gln, Arg, His, Phe, Cys, Trp, Tyr, Met, and Pro), and other amino acids that are natural products, such as norleucine, ethylglycine, ornithine, methylbutenyl-methylthreonine, and phenylglycine. Examples of amino acid side chains (e.g., R⁵) include H (glycine), methyl (alanine), -CH₂C(O)NH₂ (asparagine), -CH₂-SH (cysteine), and -CH(OH)CH₃ (threonine).

A long chain N-alkylated compound can be prepared by reductive alkylation of an amino (or imino) compound. For example, the amino or imino compound can be exposed to a long chain aldehyde, along with a reducing agent (e.g., sodium cyanoborohydride) to N-alkylate the amine. Similarly, a long chain N-oxa-alkylated compound can be prepared by reductive alkylation of an amino (or imino) compound. For example, the amino or imino compound can be exposed to a long chain oxa-aldehyde, along with a reducing agent (e.g., sodium cyanoborohydride) to N-oxa-alkylate the amine.

The compounds can include protecting groups. Various protecting groups are well known. In general, the species of protecting group is not critical, provided that it is stable to

the conditions of any subsequent reaction(s) on other positions of the compound and can be removed at the appropriate point without adversely affecting the remainder of the molecule. In addition, a protecting group may be substituted for another after substantive synthetic transformations are complete. Clearly, where a compound differs from a compound disclosed herein only in that one or more protecting groups of the disclosed compound has been substituted with a different protecting group, that compound is within the invention. Further examples and conditions are found in Greene, *Protective Groups in Organic Chemistry*, (1st Ed., 1981, Greene & Wuts, 2nd Ed., 1991).

The compounds can be purified, for example, by crystallization or chromatographic methods. The compound can be prepared stereospecifically using a stereospecific amino or imino compound as a starting material.

The amino and imino compounds used as starting materials in the preparation of the long chain N-alkylated compounds are commercially available (Sigma, St. Louis, MO; Cambridge Research Biochemicals, Norwich, Cheshire, United Kingdom; Toronto Research Chemicals, Ontario, Canada) or can be prepared by known synthetic methods. For example, the compounds can be long chain N-alkylated imino sugar compounds or oxa-substituted derivatives thereof. The imino sugar can be, for example, deoxygalactonojirimycin (DGJ), 1-methyl-deoxygalactonojirimycin (MeDGJ), deoxynorjirimycin (DNJ), altrostatin, 2R,5R-dihydroxymethyl-3R,4R-dihydroxypyrrolidine (DMDP), or derivatives, enantiomers, or stereoisomers thereof.

The syntheses of a variety of imino sugar compounds have been described. For example, methods of synthesizing DNJ derivatives are known and are described, for example, in U.S. Patent Nos. 5,622,972, 5,200,523, 5,043,273, 4,994,572, 4,246,345, 4,266,025, 4,405,714, and 4,806,650, and U.S. patent application 07/851,818, filed March 16, 1992. Methods of synthesizing other imino sugar derivatives are known and are described, for example, in U.S. Patent Nos. 4,861,892, 4,894,388, 4,910,310, 4,996,329, 5,011,929, 5,013,842, 5,017,704, 5,580,884, 5,286,877, and 5,100,797. The enantiospecific synthesis of 2R,5R-dihydroxymethyl-3R,4R-dihydroxypyrrolidine (DMDP) is described by Fleet & Smith (*Tetrahedron Lett.* 26:1469-1472, 1985).

The substituents on the imino sugar compound can influence the potency of the compound as an antiviral agent and additionally can preferentially target the molecule to one organ rather than another. Methods for comparing the potencies of various substituted compounds are provided in the Examples.

With the exception of the pyridinium compounds, which are in salt form, the compounds described herein may be used in the free amine form or in a pharmaceutically acceptable salt form. The counter anion of the pyridinium compound can be chloride, tartrate, phosphate, or sulfate. Pharmaceutical salts and methods for preparing salt forms are provided by Berge et al. (*J. Pharm. Sci.* 66:1-18, 1977). Pharmaceutically acceptable salts can be preferred for compounds that are difficult to solubilize in the pharmaceutical composition (e.g., compounds having longer alkyl chains). A salt form is illustrated, for example, by the HCl salt of an amino derivative. The compounds may also be used in the form of prodrugs, such as the 6-phosphorylated DNJ derivatives described in U.S. Patents Nos. 5,043,273 and 5,103,008. Use of compositions which further comprise a pharmaceutically acceptable carrier and compositions which further comprise components useful for delivering the composition to an animal are explicitly contemplated. Numerous pharmaceutically acceptable carriers useful for delivering the compositions to a human and components useful for delivering the composition to other animals such as cattle are known in the art. Addition of such carriers and components to the composition of the invention is well within the level of ordinary skill in the art. For example, the compounds can be di- or tetra- acetates, propionates, butyrates, or isobutyrate. The compound can be a solvate.

The invention also encompasses isotopically-labeled counterparts of compounds disclosed herein. An isotopically-labeled compound of the invention has one or more atoms replaced with an isotope having a detectable particle- or x-ray-emitting (radioactive) nucleus or a magnetogyric nucleus. Examples of such nuclei include ^2H , ^3H , ^{13}C , ^{15}N , ^{19}F , ^{29}Si , ^{31}P , ^{32}P and ^{125}I . Isotopically-labeled compounds of the invention are particularly useful as probes or research tools for spectrometric analyses, radioimmunoassays, binding assays based on scintillation, fluorography, autoradiography, and kinetic studies such as inhibition studies or determination of primary and secondary isotope effects.

The nitrogen-containing virus-inhibiting compound can be administered to a cell or an individual affected by a virus. The compound can inhibit morphogenesis of the virus, or it can treat the individual. The treatment can reduce, abate, or diminish the virus infection in the animal. For example, the N-nonyl, N-decyl, N-3-oxa-nonyl, N-3-oxa-decyl, N-7-oxa-nonyl, and N-7-oxa-decyl compounds are antiviral. The antiviral activity is substantially unrelated to the remaining functionalities of the compound.

The nitrogen-containing virus-inhibiting compound combined with at least one other antiviral compound, such as an inhibitor of a viral DNA or RNA polymerase and/or protease,

and/or at least one inhibitor of expression of viral genes, replication of the viral genome, and/or assembly of a viral particle. The supplemental antiviral compound may be any antiviral agent, which is presently recognized, or any antiviral agent which becomes recognized. By way of example, the supplemental antiviral compound may be interferon-
5 alpha, interferon-beta, ribavirin, lamivudine, brefeldin A, monensin, TUVIRUMAB™ (Protein Design Labs) PENCICLOVIR™ (SmithKline Beecham), FAMCICLOVIR™ (SmithKline Beecham), BETASERON™ (Chiron), THERADIGM-HBV™ (Cytel), Adefovir Dipivoxil (GS 840, Gilead Sciences), INTRON A™ (Schering Plough), ROFERON™ (Roche Labs), BMS 200,475 (Bristol Myers Squibb), LOBUCAVIR™ (Bristol Myers Squibb), FTC
10 (Triangle Pharmaceuticals), DAPD (Triangle Pharmaceuticals), thymosin alpha peptide, Glycovir (Block et al., *Proc. Natl. Acad. Sci. USA* 91:2235-2240, 1994), granulocyte macrophage colony stimulating factor (Martin et al., *Hepatology* 18:775-780, 1993), an "immune-cytokine" (Guidotti et al., *J. Virol.* 68:1265-1270, 1994), CDG (Fourel et al., *J. Virol.* 68:1059-1065, 1994), or the like.

15 Long chain N-alkyl compounds are agents that exhibit an inhibitory effect on viral expression. While certain short chain N-alkyl derivatives of imino sugars (e.g., N-butyl DNJ) are potent inhibitors of the N-linked oligosaccharide processing enzymes, such as α -glucosidase I and α -glucosidase II (Saunier et al., *J. Biol. Chem.* 257:14155-14161, 1982; Elbein, *Ann. Rev. Biochem.* 56:497-534, 1987). Some long chain N-alkyl compounds of the
20 invention may exhibit substantially little or no inhibition of a glycosidase enzyme, especially in comparison with N-butyl DNJ or N-nonyl DNJ. Unexpectedly, some long chain N-alkyl compounds do effectively inhibit viral morphogenesis in cells infected with a virus, such as a flavivirus or pestivirus. For example, the nitrogen-containing virus-inhibiting compound can have an IC_{50} of about 10 μ M or less, preferably about 3 μ M or less, for the inhibition of
25 BVDV or another virus, but the same compounds may exhibit little activity against glycosidases or inhibition of glycolipid synthesis.

Methods for treating a mammal infected with respiratory syncytial virus (RSV) using DNJ derivatives have been described in U.S. Patent No. 5,622,972. The use of DNJ and N-methyl-DNJ has also been disclosed to interrupt the replication of non-defective
30 retroviruses such as human immunodeficiency virus (HIV), feline leukemia virus, equine infectious anemia virus, and lentiviruses of sheep and goats (U.S. Patent Nos. 5,643,888 and 5,264,356; Acosta et al., *Am. J. Hosp. Pharm.* 51:2251-2267, 1994).

In the absence of a suitable cell culture system able to support replication of human

HCV, bovine viral diarrhea virus (BVDV) serves as the FDA approved model organism for HCV, as both share a significant degree of local protein region homology (Miller & Purcell, *Proc. Natl. Acad. Sci. USA* 87:2057-2061, 1990), common replication strategies, and probably the same subcellular location for viral envelopment. Compounds found to have an antiviral effect against BVDV are highly recommended as potential candidates for treatment of HCV.

The cytotoxicity resulting from exposure of mammalian cells in tissue culture to bovine viral diarrhea virus (BVDV) is prevented by addition of a nitrogen-containing virus-inhibiting compound to the tissue culture medium. The virus inhibitors that were used in the examples below included long chain N-alkyl derivatives of DGJ. Because BVDV is an accepted tissue culture model of HCV (Henzler & Kaiser, *Nature Biotechnology* 16:1077-1078, 1998), the compositions and methods described herein for inhibiting morphogenesis of BVDV are also useful for inhibiting morphogenesis of HCV.

The amount of antiviral agent administered to an animal or to an animal cell according to the methods of the invention is an amount effective to inhibit the viral morphogenesis from the cell. The term "inhibit" as used herein refers to the detectable reduction and/or elimination of a biological activity exhibited in the absence of a nitrogen-containing virus-inhibiting compound according to the invention. The term "effective amount" refers to that amount of composition necessary to achieve the indicated effect. The term "treatment" as used herein refers to reducing or alleviating symptoms in a subject, preventing symptoms from worsening or progressing, inhibition or elimination of the causative agent, or prevention of the infection or disorder in a subject who is free therefrom.

Thus, for example, treatment of viral infection includes destruction of the infecting agent, inhibition of or interference with its growth or maturation, neutralization of its pathological effects, and the like. The amount of the composition which is administered to the cell or animal is preferably an amount that does not induce any toxic effects which outweigh the advantages which accompany its administration.

Actual dosage levels of active ingredients in the pharmaceutical compositions of this invention may be varied so as to administer an amount of the active compound(s) that is effective to achieve the desired therapeutic response for a particular patient.

The selected dose level will depend on the activity of the selected compound, the route of administration, the severity of the condition being treated, and the condition and prior medical history of the patient being treated. However, it is within the skill of the art to

start doses of the compound(s) at levels lower than required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. If desired, the effective daily dose may be divided into multiple doses for purposes of administration, for example, two to four doses per day. It will be understood, however, that the specific dose level for any particular patient will depend on a variety of factors, including the body weight, general health, diet, time and route of administration and combination with other drugs and the severity of the disease being treated. It is expected that the adult human daily dosage will normally range from between about one microgram to about one gram, preferably from between about 10 mg and 100 mg, of the nitrogen-containing virus-inhibiting compound per kilogram body weight. Of course, the amount of the composition which should be administered to a cell or animal is dependent upon numerous factors well understood by one of skill in the art, such as the molecular weight of the nitrogen-containing virus-inhibiting compound, the route of administration, and the like.

Pharmaceutical compositions that are useful in the methods of the invention may be administered systemically in oral solid formulations, ophthalmic, suppository, aerosol, topical or other similar formulations. For example, it may be in the physical form of a powder, tablet, capsule, lozenge, gel, solution, suspension, syrup, or the like. In addition to the nitrogen-containing virus-inhibiting compound, such pharmaceutical compositions may contain pharmaceutically-acceptable carriers and other ingredients known to enhance and facilitate drug administration. Other possible formulations, such as nanoparticles, liposomes, resealed erythrocytes, and immunologically based systems may also be used to administer the compound according to the method of the invention. Such pharmaceutical compositions may be administered by any known route. The term "parenteral" used herein includes subcutaneous, intravenous, intraarterial, intrathecal, and injection and infusion techniques, without limitation. By way of example, the pharmaceutical compositions may be administered orally, topically, parenterally, systemically, or by a pulmonary route.

These compositions may be administered according to the methods of the invention in a single dose or in multiple doses which are administered at different times. Because the inhibitory effect of the composition upon a virus may persist, the dosing regimen may be adjusted such that virus propagation is retarded while the host cell is minimally effected. By way of example, an animal may be administered a dose of the composition of the invention once per week, whereby virus propagation is retarded for the entire week, while host cell functions are inhibited only for a short period once per week.

The following specific examples are to be construed as merely illustrative, and not limitative, of the remainder of the disclosure.

EXAMPLES

5 Preparation of N-nonyl-DGJ (NN-DGJ), N-nonyl-methylDGJ (NN-MeDGJ), N-nonyl-altrostatin, N-nonyl-DNJ (NN-DNJ), N-nonyl-DMDP (NN-DMDP), and N-nonyl-2-aminobenzamide

The parent amino or imino compound (DGJ, MeDGJ, altrostatin, DNJ, DMDP, or 2-aminobenzamide (2ABC9) was reductively alkylated with nonylaldehyde (1.2 mol equivalents) in the presence of one mole equivalent of sodium cyanoborohydride for three hours at room temperature in acidified methanol. Typical yields from this reaction were greater than 95% as determined by amperometric detection after high performance cation-exchange chromatography (Dionex). N-Nonyl-compounds were purified from the reaction mixture by high performance liquid chromatography (HPLC) as follows. A sample was applied to a SCX cation-exchange column (7.5 x 50 mm) in 20% (v/v) acetonitrile and eluted with a linear gradient of 20% acetonitrile containing 500 mM ammonium formate, pH 4.4. The N-nonyl compound was recovered and applied to a C18 reverse-phase column (4.6 x 250 mm) equilibrated with 10% acetonitrile containing 0.1% trifluoroacetic acid (TFA). The compound was eluted from the column using a linear gradient of 80% acetonitrile containing 0.1% trifluoroacetic acid, lyophilized to dryness, and dissolved in methanol. Samples of purified compound were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using 2,5-dihydroxybenzoic acid as the matrix.

Compounds having different N-alkyl chain lengths are prepared by replacing nonyl aldehyde with the desired chain length aldehyde. Tritiated compounds are prepared by employing tritiated sodium cyanoborohydride as the reducing agent in the reaction.

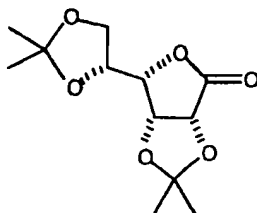
- (a) N-nonyl-DGJ: MALDI-TOF mass spectrometry showed a peak at 288.83 atomic mass units as expected for the structure shown in Figure 1.
- (b) N-nonyl-MeDGJ: MALDI-TOF mass spectrometry showed a peak at 273.9 atomic mass units as expected for the structure shown in Figure 1.
- 30 (c) N-nonyl-altrostatin: MALDI-TOF mass spectrometry showed a peak at 289.44 atomic mass units as expected for the structure shown in Figure 1.
- (d) N-nonyl-DMDP: MALDI-TOF mass spectrometry showed a peak at 287.66 atomic mass units as expected for the structure shown in Figure 1.
- (e) N-nonyl-2-aminobenzamide (2ABC9): MALDI-TOF mass spectrometry showed a

peak at 261.57 atomic mass units as expected for the structure shown in Figure 1.

Preparation of N-(7-oxa-nonyl)-1,5,6-trideoxy-1,5-imino-D-galactitol

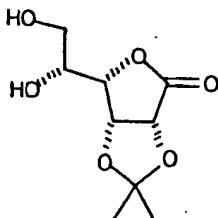
Step1: Synthesis of 2,3,5,6-Di-*O*-isopropylidene-D-gulono-1,4-lactone

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- p*-Toluenesulfonic acid-monohydrate (1 g) was added to a stirred solution of D-gulono-lactone (20 g, 0.11 mol) in 2,2-dimethoxypropane (60 mL) and dry acetone (200 mL). After 24 hr t.l.c. (ethyl acetate) showed the consumption of starting material (R_f 0.0) and the formation of a major product (R_f 0.8). The reaction mixture was neutralized by stirring with excess sodium hydrogen carbonate, filtered and the solvent removed under reduced pressure. The residue was crystallized from ethyl acetate/hexane to give 2,3,5,6-Di-*O*-isopropylidene-D-gulono-1,4-lactone as white crystals (26.3 g, 0.1 mol, 91% yield).
- 15 M.p. 150-153°C; $[\alpha]_D^{22} +76.2$ (c, 0.88 in acetone); δ_H (200 MHz, $CDCl_3$): 1.28 (s, 6H, $C(CH_3)_2$), 1.33, 1.37 (2 x s, 6H, $C(CH_3)_2$), 3.90 (dd, 1H, J 6.0 Hz, J 9.0 Hz), 4.02 - 4.10 (m, 1H), 4.18 - 4.27 (m, 1H), 4.49 (dd, 1H, $J_{3,4}$ 3 Hz, $J_{4,5}$ 9 Hz, H-4), 4.92 (dd, 1H, $J_{2,3}$ 6 Hz, $J_{3,4}$ 3 Hz, H-3), 4.96 (d, 1H, $J_{2,3}$ 6 Hz, H-2); δ_C (50 MHz, $CDCl_3$): 25.6 ($C(CH_3)_2$), 26.3 ($C(CH_3)_2$), 27.1 ($C(CH_3)_2$), 27.2 ($C(CH_3)_2$), 65.6 (CH_2 , C-2), 75.7, 76.4, 76.5, 81.3 (4 x CH, C-2, C-3, C-4), 110.9 ($C(CH_3)_2$), 114.7 ($C(CH_3)_2$), 173.3 (C=O).
- 20

Step 2: Synthesis of 2,3-*O*-isopropylidene-D-gulono-lactone



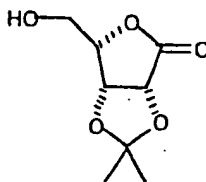
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2,3,5,6-Di-*O*-isopropylidene-D-gulono-1,4-lactone (26 g, 0.1 mol) was dissolved in aqueous

acetic acid (200 ml, 80%) and the solution was stirred overnight at room temperature. T.l.c. (ethyl acetate) showed the consumption of starting material (R_f 0.8) and the formation of one major product (R_f 0.4). The reaction solvent was removed and the residue crystallized from ethyl acetate/hexane to give 2,3-*O*-isopropylidene-D-gulono-1,4-lactone (20.7 g, 95 mmol, 95%) as a white solid.

M.p. 139-141°C; $[\alpha]_D^{22} +73.1$ (c, 2.4 in acetone); δ_H (200 MHz, $CDCl_3$): 1.21, 1.22 (2 x s, 6H, $C(CH_3)_2$), 3.46-3.57 (m, 2H), 3.64-3.73 (m, 1H), 4.48 (dd, 1H, $J_{3,4}$ 5 Hz, $J_{4,5}$ 3 Hz, H-4), 4.75 (d, 1H, $J_{2,3}$ 5 Hz, H-2), 4.81 (dd, 1H, $J_{2,3}$ 5 Hz, $J_{3,4}$ 3 Hz, H-3); δ_C (50 MHz, $CDCl_3$): 26.0 ($C(CH_3)_2$), 26.1 ($C(CH_3)_2$), 62.7 (CH_2 , C-6), 71.3 (CH, C-3), 76.7, 77.1 (2 x CH, C-4, C-5), 81.8 (CH, C-2), 113.9 ($C(CH_3)_2$), 175.5 (C=O).

Step 3: Synthesis of 2,3-*O*-isopropylidene-L-lyxono-1,4-lactone

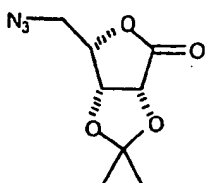


2,3-*O*-isopropylidene-D-gulonolactone (10.9 g, 50 mmol) was dissolved in dry THF (250 mL) under N_2 . Periodic acid (12.8 g, 56 mmol, 1.12 eq) was added. After 5 min, the solution became cloudy and was vigorously stirred for another 15 min. The reaction mixture was purified by elution through a silica plug eluted with ethyl acetate. The solvent was removed under reduced pressure to afford a yellow oil which was dissolved in acetic acid (150 mL). Sodium cyanoborohydride (3.22 g, 51 mmol) was added and the solution stirred for 90 min. Saturated aqueous ammonium chloride solution (20 mL) was added to quench the reaction mixture and the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (200 mL) and washed with saturated aqueous ammonium chloride solution (50 mL), water (50 mL) and brine (50 mL). The aqueous layer was re-extracted with ethyl acetate (3 x 50 mL). The organic fractions were combined, dried (magnesium sulphate), filtered and the solvent removed. Purification by flash chromatography (ethyl acetate) gave 2,3-*O*-isopropylidene-L-lyxono-1,4-lactone (7.93 g, 42 mmol, 84% yield) as a white crystalline solid.

M.p. 94-95°C; $[\alpha]_D^{23} -90.8$ (c, 1.08 in acetone); δ_H (500 MHz, $CDCl_3$): 1.41, 1.49 (6H, 2 x s,

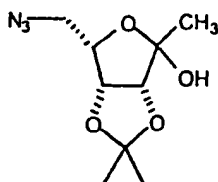
C(CH₃)₂), 2.18 (1H, br, OH), 3.87 (1H, dd, J_{4,5'} 5.3 Hz, J_{5,5'} 12.3 Hz, H-5'), 4.15 (1H, dd, J_{4,5} 6.4 Hz, J_{5,5'} 12.3 Hz, H-5), 4.56 (1H, ddd, J_{4,5'} 5.3 Hz, J_{4,5} 6.6 Hz, J_{3,4} 3.6 Hz, H-4), 4.82 (1H, d, J_{2,3} 5.5 Hz, H-2), 4.85 (1H, dd, J_{3,4} 3.6 Hz, J_{2,3} 5.5 Hz, H-3), δ_C (50 MHz, CDCl₃): 26.2 (C(CH₃)₂), 27.1 (C(CH₃)₂), 61.3 (CH₂, C-5), 76.6, 76.7, 79.8 (3 x CH, C-2, C-3, C-4), 114.9 (C(CH₃)₂), 174.3 (C=O).

Step 4: Synthesis of 5-azido-5-deoxy-2,3-*O*-isopropylidene-L-lyxono-1,4-lactone



2,3-*O*-isopropylidene-L-lyxono-1,4-lactone (5.8 g, 30.9 mmol) was dissolved in anhydrous dichloromethane (140 mL) under N₂. The solution was cooled to -30°C and dry pyridine (12 mL) was added. Trifluoromethanesulphonic anhydride (6.5 ml, 38.7 mmol) was then added dropwise to the solution which was stirred at -30°C. After 60 min, t.l.c. (ethyl acetate/hexane 1:1) showed a complete reaction. The solution was allowed to warm to 0°C and dry DMF (250 ml) and sodium azide (8.2 g, 126 mmol, 4 eq) were added. The suspension was stirred at room temperature for 4 Water (25 mL) was added to quench the reaction. The solvent was then removed under reduced pressure and co-evaporated with toluene. The residue was dissolved in dichloro methane (250 mL) and washed water (2 x 50 mL) and brine (50 mL). The aqueous layer was re-extracted with dichloro methane (3 x 50 mL). The organic fractions were combined, dried (magnesium sulphate), filtered and the solvent removed. Purification by flash chromatography (hexane/ethyl acetate 1:1) afforded 5-azido-5-deoxy-2,3-*O*-isopropylidene-L-lyxono-1,4-lactone (5.8 g, 27.2 mmol, 88% yield) as white crystals.

[α]_D²³ -71.0 (c, 2.0 in CHCl₃); ν_{max} (film/cm⁻¹) 1784 (C=O), 2101 (N₃); δ_H (500 MHz, CDCl₃): 1.42, 1.50 (6H, 2 x s, C(CH₃)₂), 3.66 (1H, dd, J_{4,5'} 6.3 Hz, J_{5,5'} 12.9 Hz, H-5'), 3.72 (1H, dd, J_{4,5} 7.1 Hz, J_{5,5'} 12.9 Hz, H-5), 4.62 (1H, ddd, J_{4,5'} 6.3 Hz, J_{4,5} 7.1 Hz, J_{3,4} 3.5 Hz, H-4), 4.83 (1H, dd, J_{3,4} 3.5 Hz, J_{2,3} 5.4 Hz, H-3), 4.86 (1H, d, J_{2,3} 5.4 Hz, H-2); δ_C (50 MHz, CDCl₃): 26.3 (C(CH₃)₂), 26.5 (C(CH₃)₂), 50.4 (CH₂, C-5), 76.1, 76.4, 77.6 (3 x CH, C-2, C-3, C-4), 115.1 (C(CH₃)₂), 173.4 (s, C=O); *m/z* (CI, NH₃): 218 (100%), 186 (35%, MH⁺-N₂); (Found: C, 45.26; H, 5.43; N, 19.24. C₈H₁₁O₄N₃ requires: C, 45.07; H, 5.20; N, 19.71%).

Step 5: Synthesis of 6-Azido-1,6-dideoxy-3,4-*O*-isopropylidene-L-lyxo-2,5-hexulose

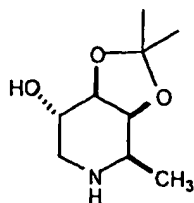
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5-Azido-5-deoxy-2,3-*O*-isopropylidene-L-lyxono-1,4-lactone (4 g, 18.8 mmol) was dissolved in dry THF (70 mL) under N₂ in presence of molecular sieves (4Å). The solution was cooled to -78°C. Methyl lithium (18 ml, 25.2 mmol, 1.4 M solution in diethyl ether) was added and the solution stirred at -78°C. After two hours, t.l.c. (ethyl acetate/hexane 1:1) showed no
 10 starting material (R_f 0.62) and a new product (R_f 0.72). Saturated aqueous ammonium chloride solution (10 mL) was added and the solution was stirred for 30 min. The reaction mixture was then extracted with dichloromethane (4 x 50 mL). The organic extracts were combined, dried (magnesium sulphate), filtered off and the solvent removed under reduced pressure. The resulting yellow solid was purified by flash chromatography (ethyl
 15 acetate/hexane 1:2) to give 6-azido-1,6-dideoxy-3,4-*O*-isopropylidene-L-lyxo-2,5-hexulose (3.49 g, 91% yield) as a white solid.

M.p. 89-90°C; $[\alpha]_D^{21}$ -12.5 (c, 1.01 in CHCl₃); ν_{\max} (KBr)/cm⁻¹: 3436 (br, OH), 2101 (N₃); δ_H (500 MHz, CDCl₃): 1.33, 1.48 (6H, 2 x s, C(CH₃)₂), 1.54 (3H, s, CH₃), 2.13 (1H, br, OH), 3.54 (2H, d, $J_{6,6}$ 6.4 Hz, H-6, H-6'), 4.23 (1H, app. dt, $J_{5,4}$ 3.9 Hz, $J_{5,6}$ 6.4 Hz, H-5), 4.48 (1H, d, $J_{3,4}$ 5.9 Hz, H-3), 4.78 (1H, dd, $J_{4,3}$ 5.9 Hz, $J_{4,5}$ 3.9 Hz, H-4); δ_C (50 MHz, CDCl₃): 22.9 (CH₃, C-1), 25.2, 26.5 (2 x CH₃, C(CH₃)₂), 50.4 (CH₂, C-6), 77.9, 80.9, 85.8 (3 x CH, C-3, C-4, C-5), 105.9 (C-2), 113.4 (C(CH₃)₂); m/z (APCI⁺): 216 (92%), 202 (MH⁺-N₂, 38%), 184 (MH⁺-H₂O-N₂, 100%); (Found: C, 47.38; H, 6.53; N, 18.03%; C₉H₁₅O₄N₃ requires C, 47.16; H, 6.60; N, 18.33%).

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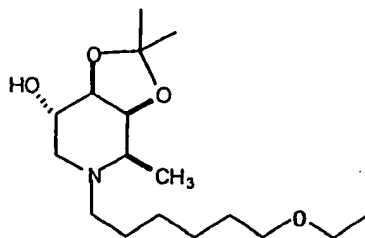
Step 6: Synthesis of 1,5,6-trideoxy-1,5-imino-3,4-*O*-isopropylidene-D-galactitol



6-Azido-1,6-dideoxy-3,4-*O*-isopropylidene-*L*-lyxo-2,5-hexulose (1.0 g, 4.4 mmol) was dissolved in ethanol (25 mL). Palladium black (300 mg) was added. The solution was degased 3 times and air was replaced by H₂. The solution was stirred at room temperature under an atmosphere of H₂. After 24 hr, the solution was filtered through a celite plug eluted with ethanol. The solvent was removed under reduced pressure to give a yellow solid which was purified by flash chromatography (chloroform/methanol 4:1) to afford 1,5,6-trideoxy-1,5-imino-3,4-*O*-isopropylidene-*D*-galactitol as a white solid (700 mg, 3.7 mmol, 84% yield).

M.p. 164-166°C; $[\alpha]_D^{22} +84.0$ (c, 1.01 in CHCl₃); ν_{\max} (cm⁻¹): 3434 (br, OH, NH); δ_H (500 MHz, CDCl₃): 1.27 (3H, d, $J_{5,6}$ 6.3 Hz, CH₃), 1.38, 1.55 (6H, 2 x s, C(CH₃)₂), 1.95 (1H, br, OH), 2.48 (1H, dd, $J_{1a,2}$ 10.6 Hz, $J_{1e,1a}$ 13.0 Hz, H-1a), 3.08 (1H, dq, $J_{4,5}$ 2.6 Hz, $J_{5,6}$ 6.3 Hz, H-5), 3.12 (1H, dd, $J_{1e,2}$ 5.1 Hz, $J_{1a,1e}$ 13.0 Hz, H-1e), 3.67 (1H, ddd, $J_{1',2}$ 5.1 Hz, $J_{1,2}$ 10.6 Hz, $J_{2,3}$ 7.1 Hz, H-2), 3.88 (1H, dd, $J_{2,3}$ 7.1 Hz, $J_{3,4}$ 5.3 Hz, H-3), 4.04 (1H, dd, $J_{4,5}$ 2.6 Hz, $J_{3,4}$ 5.3 Hz, H-4); δ_C (50 MHz, CDCl₃): 18.0 (CH₃, C-6), 26.7, 28.7 (2 x CH₃, C(CH₃)₂), 48.7 (CH₂, C-1), 51.6 (CH, C-5), 71.1, 77.0, 80.5 (3 x CH, C-2, C-3, C-4), 109.5 (C(CH₃)₂); m/z (APCI⁺): 188 (MH⁺, 100%), 130 (19%); (Found: C, 57.26; H, 9.40; N, 7.24%. C₉H₁₇O₃N requires C, 57.73; H, 9.15; N, 7.48%)

Step 7: Synthesis of *N*-nonyl-1,5,6-trideoxy-1,5-imino-3,4-*O*-isopropylidene *D*-galactitol

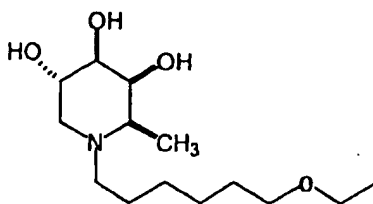


1,5,6-trideoxy-1,5-imino-3,4-*O*-isopropylidene-*D*-galactitol (804 mg, 4.3 mmol) was

dissolved in ethanol (15 mL). Glacial acetic acid (0.1 mL) and 6-ethoxy-hexanol (1.83 g, 12.9 mmol, 2.2 ml, 3 eq) were added. After stirring the reaction mixture for 20 min at room temperature under N₂. Palladium black (300 mg) was added. The solution was degassed three times and nitrogen was replaced by H₂. The solution was stirred at room temperature under an atmosphere of H₂. After 16 h, the solution was filtered through a celite plug eluted with ethanol (50 mL) and ethyl acetate (40 mL). The solvent was removed under reduced pressure to give a yellow solid which was purified by flash chromatography (ethyl acetate) to afford *N*-nonyl-1,5,6-trideoxy-1,5-imino-3,4-*O*-isopropylidene-D-galactitol as a white solid (829 mg, 2.7 mmol, 63% yield).

- 10 M.p. 41 - 43°C; δ_H (200 MHz, CDCl₃): 0.99 (3H, t, J 7.3 Hz, CH₃), 1.22 - 1.51 (15H, 6 x CH₂, CH₃, C-6), 1.35, 1.53 (6H, 2 x s, C(CH₃)₂), 2.32 (1H, t, J 10.3 Hz, H-1a), 2.52 - 2.96 (m, 3H, H-5, *N*-CH₂), 3.82 - 3.94 (2H, m, H-1e, H-4); 4.12 (1H, m, H-2); δ_C (50 MHz, CDCl₃): 14.6 (CH₃), 16.0 (CH₃, C-6), 23.1, 24.4 (2 x CH₃, C(CH₃)₂), 27.9, 29.7, 29.9, 32.3 (4 x CH₂), 53.4 (CH₂, C-1), 54.1 (CH₂, *N*-CH₂), 55.1 (CH, C-5), 70.2, 78.1, 79.7 (3 x CH, C-2, C-3, C-4), 109.6 (C(CH₃)₂);

Step 8: Synthesis of *N*-(7-oxa-nonyl)-1,5,6-trideoxy-1,5-imino-D-galactitol



20

N-nonyl-1,5,6-trideoxy-1,5-imino-3,4-*O*-isopropylidene-D-galactitol (1.4 g, 4.5 mmol) was dissolved in 50% aqueous trifluoroacetic acid (10 mL) and the solution was stirred for two hours. The solvent was removed under reduced pressure and co-evaporated with toluene (2 x 5 mL). Purification by flash chromatography (CHCl₃/CH₃OH 3:1) afforded *N*-nonyl-1,5,6-trideoxy-1,5-imino-D-galactitol (1.18 g, 4.3 mmol, 96% yield);

- 25 M.p. 49-51°C; ν_{\max} (cm⁻¹): 3434 (br, OH), 2845 (N-CH₂), 1672 (N-CH₂), 1203, 1133; δ_H (200 MHz, d⁴-MeOH): 0.99 (3H, t, J 7.3 Hz, CH₃), 1.22 - 1.51 (15H, 6 x CH₂, CH₃, C-6), 2.88 (1H, t, J 10.6 Hz, H-1a), 3.16 (2H, m, *N*-CH₂), 3.31 (1H, m, H-5), 3.42 (1H, dd, J_{1e,2} 5.0 Hz, J_{1a,1e} 10.6 Hz, H-1e), 3.51 (1H, dd, J_{4,5} 2.6 Hz, J_{3,4} 5.3 Hz, H-4); 3.91 3.51 (1H, dd, J_{4,5} 2.6 Hz, J_{3,4} 5.3 Hz, H-4); 4.08 (1H, ddd, J_{1',2} 5.1 Hz J_{1,2} 10.6 Hz, J_{2,3} 7.1 Hz, H-2), δ_C (50 MHz,
- 30

CDCl₃): 13.4 (CH₃), 13.6 (CH₃, C-6), 22.1, 22.7, 26.7, 29.3, 29.5, 32.0 (6 x CH₂), 52.9 (CH₂, N-CH₂), 54.2 CH₂, C-1), 60.9, 65.5, 71.9, 74.1 (4 x CH, C-2, C-3, C-4, C-5); *m/z* (APCI⁺): 274.2 (MH⁺, 100%).

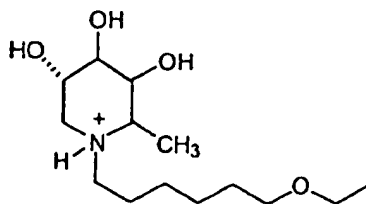
5 Preparation of N-7-oxa-nonyl-DGJ, N-7-oxa-nonyl-methylDGJ, N-7-oxa-nonyl-DMDP, and N-7-oxa-nonyl-2-aminobenzamide

The parent amino or imino compound (DGJ, MeDGJ, DMDP, or 2-aminobenzamide (2ABC9) was reductively alkylated with 6-ethoxy-hexanal (1.2 mol equivalents) in the presence of one mole equivalent of sodium cyanoborohydride for three hours at room
 10 temperature in acidified methanol. Typical yields from this reaction were greater than 95% as determined by amperometric detection after high performance cation-exchange chromatography (Dionex). N-7-oxa-nonyl-compounds were purified from the reaction mixture by high performance liquid chromatography (HPLC) as follows. A sample was applied to a SCX cation-exchange column (7.5 x 50 mm) in 20% (v/v) acetonitrile and eluted
 15 with a linear gradient of 20% acetonitrile containing 500 mM ammonium formate, pH 4.4. The N-7-oxa-nonyl compound was recovered and applied to a C18 reverse-phase column (4.6 x 250 mm) equilibrated with 10% acetonitrile containing 0.1% trifluoroacetic acid (TFA). The compound was eluted from the column using a linear gradient of 80% acetonitrile containing 0.1% trifluoroacetic acid, lyophilized to dryness, and dissolved in methanol.
 20 Samples of purified compound were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using 2,5-dihydroxybenzoic acid as the matrix.

Compounds having different N-7-oxa-alkyl chain lengths are prepared by replacing oxanonyl-aldehyde with the desired chain length aldehyde. Tritiated compounds are prepared
 25 by employing tritiated sodium cyanoborohydride as the reducing agent in the reaction.

Characterization of Synthesized Compounds

N-(7-oxa-nonyl)-1,5,6-trideoxy-1,5-imino-D-galactitol (chloride salt)



N-(7-oxa-nonyl)-1,5,6-trideoxy-1,5-imino-3,4-*O*-isopropylidene-D-galactitol (70 mg, 0.22 mmol) was dissolved in 50% aqueous trifluoroacetic acid (1 mL) and the solution was stirred for two hours. The solvent was removed under reduced pressure. Purification by flash chromatography (CHCl₃/CH₃OH 3:1) afforded *N*-(7-oxa-nonyl)-1,5,6-trideoxy-1,5-imino-D-galactitol (60 mg, 0.21 mmol, 96% yield). The compound was dissolved in water (1 mL) and aqueous hydrogen chloride solution (0.18 ml, 2M, 1 eq.) was added (pH 2). The reaction mixture was stirred for three hours, after this time t.l.c. (CHCl₃/MeOH 4:1) showed consumption of the starting material (*r_f* = 0.19) and one baseline spot. The solvent was removed under reduced pressure and the remaining solid was freeze dried for 24 hr to give a yellow solid (65 mg, 0.23 mmol, 99%). The following data is for the product prior to treatment with HCl:

δ_H (200 MHz, d⁴-MeOH): 1.15 (3H, t, J 7.1, CH₃), 1.39 (3H, d, J 6.5, CH₃, C-6), 1.45 - 1.81 (10H, 5 x CH₂), 2.92 (1H, t, J 10.6 Hz, H-1a), 3.02 - 3.18 (2H, m, H-1e, H-5); 3.22 - 3.62 (8H, m, N-CH₂, 2 x O-CH₂, H-2, H-4), 4.04 - 4.12 (2H, m, H-3, H-4); δ_C (50 MHz, CDCl₃): 13.6 (CH₃), 14.5 (CH₃, C-6), 22.0, 25.8, 26.5, 29.5 (4 x CH₂), 52.8 (CH₂, C-1), 54.2 (CH₂, N-CH₂), 61.0 (CH, C-5), 66.2, 70.4, (2 x CH₂, CH₂-O-CH₂), 65.5, 71.9, 74.1 (3 x CH, C-2, C-3, C-4); *m/z* (APCI⁺): 276.2 (MH⁺, 100%).

Toxicity of various chain length N-alkyl DNJ in MDBK cells are shown in Table 1.

TABLE 1

N-alkyl Chain Length	% Viability at 10 μ M	% Viability at 100 μ M
C ₄	74	77
C ₅	80	70
C ₆	73	71
C ₈	70	71
C ₉	56	41
C ₁₀	73	43
C ₁₂	86	1
C ₁₆	88	4
C ₁₈	84	2

The inhibitory activity (IC₅₀) and the cell cytotoxicity (IC₅₀) of various compounds, as well as their effect on α -glucosidase and ceramide glucosyl transferase, are shown in Table 2.

TABLE 2

Compound	Inhibitor of α glucosidase	Inhibitor of glycolipid synthesis	Anti-viral effect on BVDV in MDBK cells		
			IC ₅₀	CC ₅₀	Selectivity index (CC ₅₀ /IC ₅₀)
DNJ	Yes	No	Yes 20 μ M	ND	ND
N-butyl DNJ	Yes	Yes	Yes 60-120 μ M	>>10 mM	>>100
N-nonyl DNJ	Yes	Yes	Yes 2-3 μ M	250 μ M	83-125
N-butyl DGJ	No	Yes	No		
N-nonyl DGJ	No	Yes	Yes 5 μ M	250 μ M	50
N-nonyl MeDGJ	No	No	Yes 2-3 μ M	ND	ND
N-7-oxa-decyl DNJ	Yes	Yes	Yes 15-20 μ M	8 mM	400-533
N-7-oxa-nonyl MeDGJ	No	No	Yes 1.5 μ M	2.1 mM	1400

Note the lack of cell cytotoxicity of the N-alkyl oxa-substituted compound and its superior
 5 selectivity index.

Other Materials and Methods

Cells and transfection: CHO, MDBK and Hep G2 cells were grown in RPMI 1640 (Gibco-BRL, Rockville, MD) containing 10% fetal bovine serum (Gibco-BRL). Hep G2.2.15 cells
 10 were kindly provided by Dr. George Acs (Mt. Sinai Medical College (New York, NY) and maintained in the same manner as Hep G2 cells but with the addition of 200 μ g/ml of G418 (Gibco-BRL). DNA transfection of Hep G2 cells were performed as previously described (Bruss & Ganem, *Proc. Natl. Acad. Sci. USA* 88:1059-1063, 1991). N-butyl deoxynojiricmycin (NB-DNJ) was provided by Monsanto/Searle (St. Louis, MO). N-nonyl

deoxygalactojirimycin (N-nonyl-DGJ) and N-nonyl deoxynojirimycin (N-nonyl-DNJ) were provided by Synergy Pharmaceuticals (Somerset, NJ).

5 Plaque Reduction and Yield Assays: MDBK cells were grown in six-well plates in the presence or absence of inhibitor, infected with cp BVDV (moi = 0.005; 500 pfu per well) for one hour at 37°C. The inoculum was then replaced with growth medium alone or with growth media and the antiviral agent and incubated for two or three days in the presence or absence of inhibitor (plaque reduction assay). After counting the plaques by eye under the microscope, the supernatant containing secreted infectious virus was removed from the wells
10 and used to infect a fresh monolayer of MDBK cells in six-well plates. After three days, the resulting plaques were counted under the microscope (yield assay).

Figure 5 is a bar graph showing average IC₅₀ values for N-nonyl-DGJ, N-nonyl-MeDGJ, N-nonyl-DNJ, N-DMDP, N-nonyl-2-aminobenzamide (ABC9), nonylamine, and N-nonyl-altrostatin. The percent of BVDV plaques produced by an infected cell culture in the presence of different concentrations of 2ABC9 (◆), nonylamine (■), N-nonyl-altrostatin (Δ), N-nonyl-DGJ (×), N-nonyl-MeDGJ (⋈), N-nonyl-DNJ (●), and N-nonyl-DMDP (+) are shown in Figure 6. IC₅₀ values for N-nonyl-MeDGJ was less than about 2.5 μM as shown in Figure 7.

20 Secreted DNA analysis: Secreted DNA analysis was performed by the method of Wei et al. (*J. Virol.* 70:6455-6458, 1996). Hep G2.1.15 cells were seeded at 85-90% confluency in T-75 flasks and three days later the indicated drug added at the specified concentrations: 3TC (1 μM unless noted); N-butyl-DNJ (4.52 mM); N-nonyl DNJ (either 7 μM, 70 μM or 100 μM as noted); N-nonyl-DGJ (either 7 μM, 70 μM or 100 μM as noted). Media containing drug
25 was changed every two days and on the 7th day the media taken and the virus concentrated by pelleting through 20% sucrose for 16 hours (SW 41 rotor, 36,000 RPM). Virus was resuspended in 400 μL of 10 mM TRIS (pH 7.9), 10 mM EDTA (pH 8.0), and 10 mM MgCl₂. Samples were split into two 200 μL aliquots and labeled as +Dnase and -Dnase. To both tubes, 15 μl of proteinase K was added to a final concentration of 750 μg/ml for one
30 hour at 37°C. After one hour, 10 μl Dnase was added to the tube labeled +Dnase (final concentration is 50 units/ml) and incubated at 37°C for one hour. SDS was added to a final concentration of 1% and additional proteinase K added to a final concentration of 500 μg/ml and the reaction allowed to proceed at 37°C for 3-4 hours. DNA was then purified by

phenol/chloroform extraction. DNA was separated on 1% agarose gel and probed with ^{32}P labeled probes as described (Mehta et al., *Proc. Natl. Acad. Sci. USA* 94:1822-1827, 1997).

5 Intracellular DNA analysis: Hep G2.2.15 cells were either left untreated or treated with the compounds listed above for seven days and the total DNA extracted as described (Mehta et al., *ibid.*). DNA (20 μg) was digested with HindIII, resolved through a 1.2% agarose gel and transferred to nylon membranes. Membranes were then hybridized with a ^{32}P labeled probe containing the total HBV genome and developed as described (Lu et al., *Proc. Natl. Acad. Sci. USA* 94:2380-2385, 1997). The relaxed circular (rc), linear (lin), and closed circular
10 (CCC) DNA were confirmed by enzymatic digestion.

Endogenous polymerase assay: Media containing HBV from Hep G2.2.15 cells was pelleted through 20% sucrose (SW 28 Rotor, 24,000 RPM) for 16 hours and the pellet re-suspended in 50 μl of a mixture containing 50 mM Tris (pH 7.5), 75 mM NH_4Cl , 1 mM EDTA, 25 mM
15 MgCl_2 , 0.1% β -mercaptoethanol, 0.5% NP-40, 0.4 mM each of dATP, dGTP, dTTP and 10 μl of P^{32} labeled dCTP. Drug was added to a final concentration of 3TC (7 μM), NB-DNJ (5 mM), NN-DNJ (100 μM) and NN-DGJ (100 μM) and the samples placed at 37°C overnight and the next day proteinase K was added to a final concentration of 500 $\mu\text{g/ml}$ and incubated at 37°C for one hour. DNA was purified by a phenol/chloroform extraction and ethanol
20 precipitation.

Secretion of Infectious BVDV in the Presence of Long Chain N-Alkyl Compounds

MDBK cells were grown to semi-confluence in individual wells of 24-well plates. The cells were then infected by BVDV by incubating the cells for one hour at 37°C in the
25 presence of approximately 500 PFU of the NADL strain of BVDV suspended in growth medium. The inoculum was then replaced with growth medium alone or growth medium containing a particular concentration of a long chain N-alkyl compound. After three days, the supernatants were removed and used to infect fresh MDBK monolayers in six-well plates. After three days, the cell monolayers were observed microscopically before and after staining
30 with 0.2% (w/v) crystal violet in ethanol for plaque counting, and 0.2% neutral red for viability and the presence and number of virus-induced plaques was determined. The results were expressed as percentages of the number of plaques resulting from infection with the inhibitor-free plaque assay supernatant (=100%). The results of these experiments are

presented in the graphs depicted in Figure 2, Figure 3, and Figure 4. Figure 2 is a graph depicting the variation in IC_{50} for N-alkylated DNJ compounds having the following chain lengths: butyl, pentyl, hexyl, octyl, nonyl, decyl, dodecyl, hexadecyl, and octadecyl.

Inhibitory constants for various chain length N-alkyl DNJ derivatives for ceramide glucosyl transferase (CerGlcT) and α -glucosidase are summarized in Table 3.

TABLE 3

N-alkyl Chain Length	CerGlcT (IC_{50} , μ M)	α -Glucosidase (IC_{50} , μ M)
C ₄	34.4	0.57
C ₅	26.8	
C ₆	23.8	
C ₈	16.8	
C ₉	7.4	
C ₁₀	3.1	0.48
C ₁₂	5.2	
C ₁₆	3.4	
C ₁₈	4.1	

Uptake of radioactively labeled inhibitors by different cell types

MDBK and HepG2 cells were grown to confluency in 12-well plates and incubated in the presence of tritiated long chain N-alkylated compounds (100,000 cpm/well) for the times indicated in Figure 7: The supernatant was removed and kept. The cells were washed with PBS (2x500 μ L), fixed with 500 μ L of ice-cold 10% perchloric acid/2% phosphotungstic acid, washed twice with 500 μ L of icecold ethanol, air dried, and lysed overnight at room temperature with 500 μ L of 0.5 M NaOH. The percentage of radioactive counts in the supernatant, PBS wash and lysed cells was determined by liquid scintillation counting. The results are shown graphically in Figure 8.

Secretion of HBV in the presence of lamivudine, NN-DNJ and NN-DGJ

Hep G 2.2.15 cells are a stably transfected line of HepG2 hepatoblastoma cells that contain a dimer of the HBV genome and produce and secrete infectious HBV. This is a cell line that has been used as a standard in the pre-clinical evaluation of HBV antiviral agents, as enveloped HBV can be detected in the culture medium by antigen capture methods. The ability of NN-DGJ to inhibit enveloped HBV secretion from 2.2.15 cells was compared with lamivudine (3TC) and NN-DNJ, using the antigen capture method, described previously. Briefly, 2.2.15 cells were grown to confluence and then incubated with the indicated

concentrations of compound. At 6 and 9 days after incubation in the presence of compound, the amount of enveloped HBV in the culture medium was determined by PCR amplification of viral DNA from samples obtained by immunoprecipitation with HbsAg specific antibody. The results after nine days of incubation are shown in Table 4. Medium collected after nine days of incubation contained easily detectable amounts of HBV. As expected, 3TC (lamivudine) was effective in reducing the amount of enveloped HBV in the culture medium, when compared with the untreated controls. NN-DGJ was at least as effective as NN-DNJ in reducing the HBV secretion. The IC₅₀ values for NN-DNJ and NN-DGJ were about 1 and 0.5 μ M, respectively, in this assay. MTT assays of these cultures revealed that no measurable toxicity was observed for the concentrations used and time of exposure. These results showed that NN-DGJ is effective in preventing the secretion of HBV from Hep G2.2.15 cells at micromolar concentrations.

TABLE 4: Secretion of Hepatitis B virus (HBV) from Hep G2.2.15 cells in the absence and presence of antiviral compounds

COMPOUND ¹	IC 50 ²	TOX 50 ³
3TC	5 μ M	>100 μ M
NN-DNJ	0.4-4 μ M	>100 μ M
NN-DGJ	1.5-5 μ M	>200 μ M

¹2.2.15 cells were grown to confluence in 96 well trays and the amount of HBV in the culture medium determined by an antigen capture/PCR based assay after 6 and 9 days of incubation the absence or presence of three concentrations of either 3TC (lamivudine), NN-DNJ or NN-DGJ. Pairs of wells were used for each concentration point.

²IC 50: The concentration of compound that prevented the secretion of 50% of the amount of HBV detected in the medium from wells containing untreated cultures. IC 90s were achieved for each of the compounds used.

³TOX 50: The concentration of compound that reduced the amount of MTT activity to 50% of that of the untreated controls, as determined on the cultures at the conclusion of the

experiment (10 days). Note that because Tox 50s were not reached with even the highest concentrations of compounds used, values are given as ">" (more than).

Effect of N-nonyl-DGJ on secretion of HBV as measured by Southern blot hybridization

- 5 HepG2.2.15 cells were grown for seven days in the absence or presence of NB-DNJ (1000 µg/ml), NN-DNJ (20 µg/ml) or NN-DGJ (20 µg/ml), respectively. After seven days, virus was isolated from these cell cultures, concentrated, and purified. Secreted HBV DNA was detected by Southern blot hybridization. HBV viral DNA from untreated cells was readily detected. The secretion of HBV DNA from treated HepG2.2.15 was also detected.
- 10 N-butyl-DNJ and N-nonyl-DNJ caused a small decrease of about 3-fold and 1.5-fold secreted virus DNA, respectively; whereas N-nonyl-DGJ showed a considerably greater reduction of about 14-fold.

Intracellular levels of HBV DNA in HepG2.2.15 cells grown in the presence of 3TC, and various iminosugars

- 15 An infected cell contains several forms of HBV DNA which represent different stages in the HBV life cycle. For example, covalently closed circular DNA (CCC DNA) is the nuclear form of the DNA and is thought to be the viral template (Heermann & Gerlich, 1992). In contrast, the relaxed circular DNA (rc DNA) and linear forms (lin) are associated with the
- 20 viral particle and their presence is an indicator of encapsidation of the viral pre-genomic RNA and the subsequent reverse transcription into progeny DNA (Ganem, *Curr. Top. Microbiol. Immunol.* 168:61-83, 1991). The accumulation of intracellular HBV DNA from HepG2.2.15 cells left untreated or treated with 3TC (1 µg/ml), NB-DNJ (1000 µg/ml), NN-DNJ (2 µg/ml or 20 µg/ml), or NN-DGJ (2 µg/ml or 20 µg/ml) was determined as described
- 25 above. The amount of virus associated with the cells was detected seven days later by Southern blot analysis. The locations of the HBV relaxed circular DNA (rcDNA), covalently close circular (CCC) DNA, and single stranded (SS) DNA was identified by relative mobility.

- HBV relaxed circular DNA (rc DNA) is easily observed, as are the smaller replicative
- 30 intermediates. Treatment with 3TC leads to a complete disappearance of intracellular HBV DNA. This is consistent with 3TC acting as a polymerase inhibitor and preventing DNA production (Doong et al., *Proc. Natl. Acad. Sci. USA* 88:8495-8499, 1991). In contrast, treatment with N-butyl-DNJ causes a dramatic increase in the replicative forms of HBV DNA

(Mehta et al., *Proc. Natl. Acad. Sci. USA* 94:1822-1827, 1997). This finding is consistent with the action of this drug in preventing viral envelopment and budding but having no direct effect on DNA synthesis. Surprisingly, N-nonyl-DNJ did not cause a large increase in intracellular HBV DNA but rather a reduction. This reduction was even more pronounced
5 with N-nonyl-DGJ, leading to an almost complete disappearance of intracellular HBV DNA (greater than 25 fold). This result clearly differentiates the action of N-nonyl-DNJ and N-nonyl-DGJ from N-butyl-DNJ.

Effect of lamivudine and iminosugars on HepG2.2.15 polymerase activity

10 HBV DNA replication involves the conversion of a pregenomic RNA (pgRNA) into DNA by the action of the HBV polymerase. Current nucleoside analogue drugs (e.g., 3TC) for treating HBV target this reaction, preventing the formation and secretion of HBV viral DNA. Because the iminosugar N-nonyl-DGJ prevents the formation of HBV rc DNA, it was important to determine whether N-nonyl-DGJ was acting by inhibiting the elongation step of
15 the polymerase. HBV virions from normal and drug treated Hep G2.2.15 cells were purified and the endogenous polymerase activity was measured. HBV virions were purified from the culture medium of untreated cells by ultracentrifugation and the polymerase activity (in the presence of the indicated compounds) tested by the method of Ganem et al. (1998). Briefly, partially purified viral particles were incubated overnight with the indicated concentrations of
20 compound and 10 μ Ci of 32 P-dCTP. Viral DNA was purified by phenol extraction and ethanol precipitation and resolved on a 1.2% agarose gel. The gel was dried and viral DNA bands detected using a PhosphorImager.

The activity of polymerase from untreated virons was measured by incorporation of radioactive nucleotides into rc DNA. In contrast, treatment with 3TC (20 μ M) inhibited
25 polymerase activity. This is consistent with 3TC acting as a polymerase inhibitor. N-butyl-DNJ (4.52 mM) showed no effect on polymerase activity, consistent with its mechanism as an α -glucosidase inhibitor. Both N-nonyl-DNJ (69 μ M) and N-nonyl-DGJ (69 μ M) also had no effect on polymerase activity, although both these drugs were shown above to cause a significant decrease in intercellular HBV DNA levels. These data suggest that these alkyl
30 chain derivatives must inhibit the formation or stability of the HBV DNA by an alternative method than inhibition of polymerase activity.

All cited publications, books, patents, and patent applications are incorporated by reference in their entirety where they are cited including the priority documents U.S. Appln.

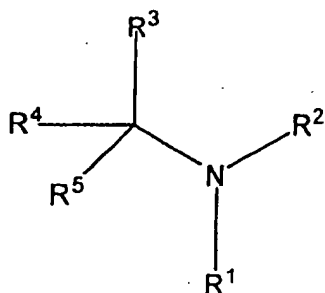
No. 60/148,101 filed August 10, 1999 and U.S. Appln. No. 60/198,621 filed April 20, 2000.

From the foregoing, it would be apparent to persons skilled in the art that the invention can be embodied in other specific forms without departing from its spirit or essential characteristics. For example, all combinations of the embodiments described above
5 are considered part of the invention with the proviso that the prior art is excluded. The described embodiments should be considered only as illustrative, not restrictive, because the scope of the invention will be indicated by the appended claims rather than by the foregoing description. All modifications which come within the meaning and range of the lawful
10 order of process steps is intended unless explicitly recited.

CLAIMS

1. A method of inhibiting morphogenesis of a pestivirus or a flavivirus comprising administering an effective amount of a nitrogen-containing virus-inhibiting compound, or a pharmaceutically acceptable salt thereof, to a cell or an individual infected with said virus, wherein said nitrogen-containing virus-inhibiting compound is comprised of an N-C₈-C₁₆ alkyl group or an oxa-substituted derivative thereof with the proviso that said nitrogen-containing virus-inhibiting compound is not N-nonyl-1,5-deoxy-1,5-imino-D-glucitol (N-nonyl-DNJ).
2. The method of claim 1, wherein the nitrogen-containing virus-inhibiting compound includes an N-C₈-C₁₀ alkyl group or an oxa-substituted derivative thereof.
3. The method of claim 2, wherein the nitrogen-containing virus-inhibiting compound is N-nonyl-1,5-dideoxy-1,5-imino-D-galactitol (N-nonyl DGJ) or N-nonyl-1,5,6-trideoxy-1,5-imino-D-galactitol (N-nonyl MeDGJ).
4. The method of claim 2, wherein the nitrogen-containing virus-inhibiting compound includes an N-oxa-nonyl group.
5. The method of any one of claims 1-4, wherein the nitrogen-containing virus-inhibiting compound is selected from the group consisting of N-alkylated piperidines, N-alkylated pyrrolidines, N-alkylated phenylamines, N-alkylated pyridines, N-alkylated pyrroles, N-alkylated amino acids, and oxa-substituted derivatives thereof.
6. The method of claim 5, wherein the nitrogen-containing virus-inhibiting compound is an N-alkylated piperidine, N-alkylated pyrrolidine, or oxa-substituted derivative thereof which is an imino sugar.
7. The method of any one of claims 1-4, wherein the nitrogen-containing virus-inhibiting compound has an IC₅₀ of about 20 μ M or less for inhibition of hepatitis B virus.

8. The method of any one of claims 1-4, wherein the nitrogen-containing virus-inhibiting compound has an IC_{50} of about 5 μM or less for inhibition of hepatitis B virus.
9. The method of any one of claims 1-4, wherein the nitrogen-containing virus-inhibiting compound has an IC_{50} of about 20 μM or less for inhibition of hepatitis B virus.
10. The method of any one of claims 1-4, wherein the nitrogen-containing virus-inhibiting compound has an IC_{50} of about 5 μM or less for inhibition of bovine viral diarrhea virus.
11. The method of any one of claims 1-10, wherein the nitrogen-containing virus-inhibiting compound does not inhibit α -glucosidase and ceramide glucosyl transferase as well as N-nonyl-DNJ.
12. The method of claim 1, wherein the nitrogen-containing virus-inhibiting compound has the formula:



wherein:

R^1 is a C_8 - C_{16} alkyl or an oxa-substituted derivative thereof;

R^2 is hydrogen, R^3 is carboxy or a C_1 - C_4 alkoxy carbonyl, or R^2 and R^3 , together, are

$$\begin{array}{c}
 X \quad Y \\
 \backslash \quad / \\
 -(C)_n- \text{ or } -(CXY)_n-, \text{ wherein } n \text{ is } 3 \text{ or } 4, \text{ each } X, \text{ independently, is selected from the group} \\
 \text{consisting of hydrogen, hydroxy, amino, carboxy, } C_1\text{-}C_4 \text{ alkylcarboxy, } C_1\text{-}C_4 \text{ alkyl, } C_1\text{-}C_4 \\
 \text{alkoxy, } C_1\text{-}C_4 \text{ hydroxyalkyl, } C_1\text{-}C_6 \text{ acyloxy, and aroyloxy, and each } Y, \text{ independently, is} \\
 \text{selected from the group consisting of hydrogen, hydroxy, amino, carboxy, } C_1\text{-}C_4
 \end{array}$$

alkylcarboxy, C₁-C₄ alkyl, C₁-C₄ alkoxy, C₁-C₄ hydroxyalkyl, C₁-C₆ acyloxy, aroyloxy, and deleted;

R⁴ is hydrogen or deleted; and

R⁵ is selected from the group consisting of hydrogen, hydroxy, amino, substituted amino, carboxy, alkoxycarbonyl, aminocarbonyl, alkyl, aryl, aralkyl, alkoxy, hydroxyalkyl, acyloxy, and aroyloxy, or R³ and R⁵, together, form a phenyl and R⁴ is deleted; wherein when R² and R³, together, are -(CXY)_n- and R⁴ is deleted, all Y are deleted, or a physiologically acceptable salt or solvate of said compound.

13. The method of claim 12, wherein R¹ is a C₈-C₁₀ alkyl or an oxa-substituted derivative thereof.

14. The method of claim 13, wherein R² is hydrogen, R³ is carboxy or C₁-C₄ alkoxycarbonyl, R⁴ is hydrogen, and R⁵ is selected from the group consisting of hydrogen, hydroxy, amino, substituted amino, carboxy, alkoxycarbonyl, aminocarbonyl, alkyl, aryl, aralkyl, alkoxy, hydroxyalkyl, acyloxy, and aroyloxy.

15. The method of claim 14, wherein R³ is carboxy.

16. The method of claim 14, wherein R³ and R⁵, together, form a phenyl and R⁴ is deleted.

17. The method of claim 12 or claim 13, wherein R² and R³, together, are -(CXY)_n-, wherein n is 3 or 4, each X and each Y, independently, is selected from the group consisting of hydrogen, hydroxy, amino, carboxy, C₁-C₄ alkylcarboxy, C₁-C₄ alkyl, C₁-C₄ alkoxy, C₁-C₄ hydroxyalkyl, C₁-C₆ acyloxy, and aroyloxy.

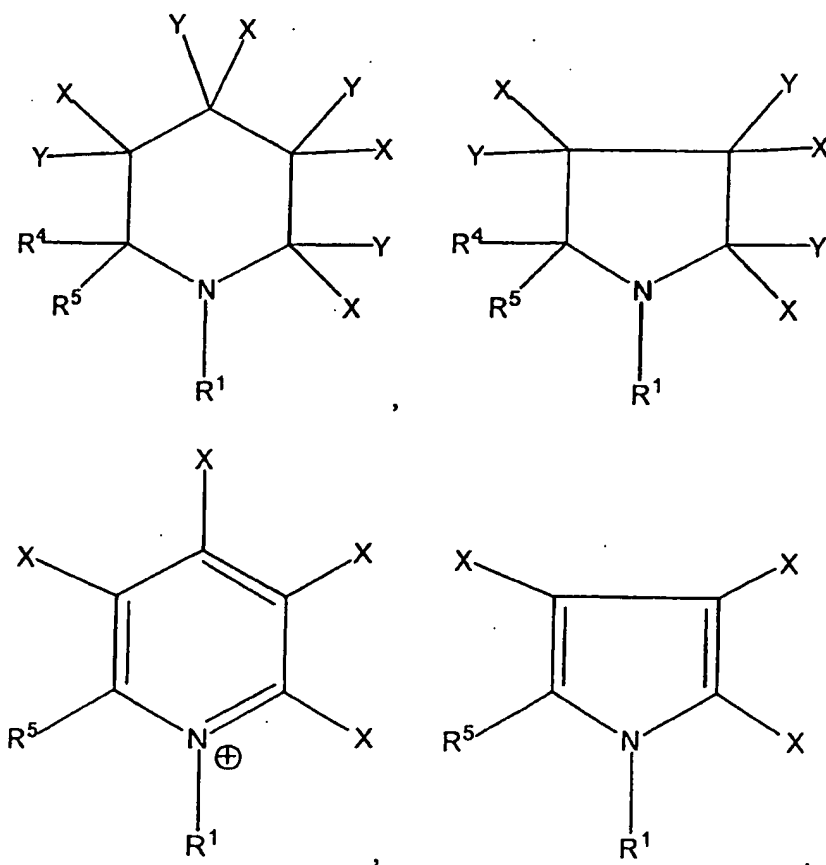
18. The method of claim 17, wherein each X is hydrogen and each Y, independently, is selected from the group consisting of hydroxy, C₁-C₄ alkyl, C₁-C₄ alkoxy, C₁-C₄ hydroxyalkyl, C₁-C₆ acyloxy, and aroyloxy.

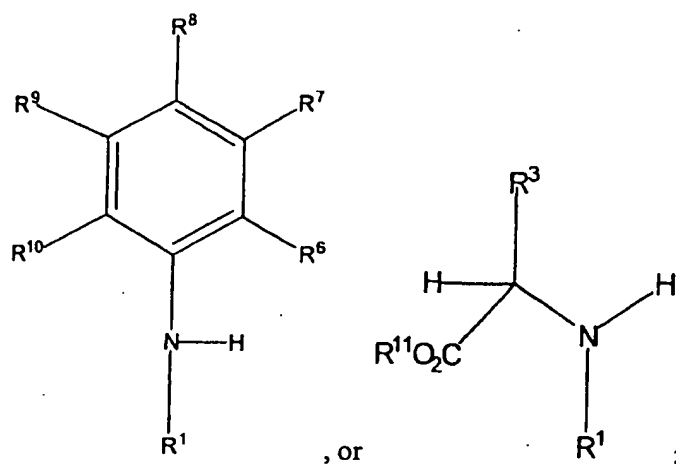
19. The method of claim 18, wherein R⁴ is hydrogen and R⁵ is hydrogen.

20. The method of claim 13, wherein R^4 is deleted and R^2 and R^3 , together, are $-(CXY)_n-$, wherein n is 3 or 4, each Y is deleted, and each X , independently, is selected from the group consisting of hydrogen, hydroxy, amino, carboxy, C_1 - C_4 alkylcarboxy, C_1 - C_4 alkyl, C_1 - C_4 alkoxy, C_1 - C_4 hydroxyalkyl, C_1 - C_6 acyloxy, and aroyloxy.

21. The method of claim 13, wherein each X , independently, is selected from the group consisting of hydrogen, hydroxy, C_1 - C_4 alkyl, C_1 - C_4 alkoxy, C_1 - C_4 hydroxyalkyl, C_1 - C_6 acyloxy, and aroyloxy.

22. The method of claim 12, wherein the nitrogen-containing virus-inhibiting compound has the formula:

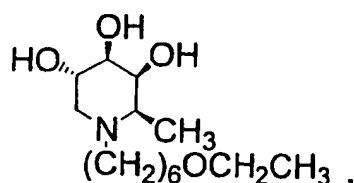




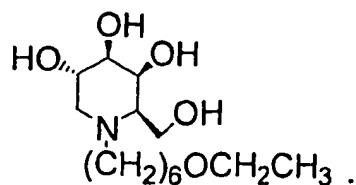
wherein each of R⁶-R¹⁰, independently, is selected from the group consisting of hydrogen, hydroxy, amino, carboxy, C₁-C₄ alkylcarboxy, C₁-C₄ alkyl, C₁-C₄ alkoxy, C₁-C₄ hydroxyalkyl, C₁-C₄ acyloxy, and aryloxy; and R¹¹ is hydrogen or C₁-C₆ alkyl.

23. The method of claim 1, wherein the nitrogen-containing virus-inhibiting compound is selected from the group consisting of N-nonyl altrostatin, N-nonyl-2*R*,5*R*-dihydroxymethyl-3*R*,4*R*-dihydropyrrolidine (N-nonyl DMDP), and N-nonyl-2-aminobenzamide (2ABC9).

24. The method of claim 1, wherein the nitrogen-containing virus-inhibiting compound is N-(7-oxa-nonyl)-1,5,6-trideoxy-1,5-imino-D-galactitol (N-7-oxa-nonyl MeDGJ)



25. The method of claim 1, wherein the nitrogen-containing virus-inhibiting compound is N-(7-oxa-nonyl)-1,5-dideoxy-1,5-imino-D-galactitol (N-7-oxa-nonyl DGJ)



26. The method of any one of claims 1-25, wherein a mammalian cell is treated.

27. The method of any one of claims 1-25, wherein a human cell is treated.
28. The method of any one of claims 1-25, wherein a mammal is treated.
29. The method of any one of claims 1-25, wherein a human is treated.
30. The method of any one of claims 1-25, wherein the virus is a hepatitis B virus.
31. The method of any one of claims 1-25, wherein the virus is a hepatitis C virus.
32. A compound having the formula shown in claim 12 or a physiologically acceptable salt or solvate of said compound.
33. The compound of claim 32, wherein the compound is selected from the group consisting of N-nonyl-1,5-dideoxy-1,5-imino-D-galactitol (N-nonyl DGJ) N-nonyl-1,5,6-trideoxy-1,5-imino-D-galactitol (N-nonyl MeDGJ), and physiologically acceptable salts or solvates thereof.
34. The compound of claim 32, wherein the compound is selected from the group consisting of N-nonyl altrostatin, N-nonyl DMDP, N-nonyl-2-aminobenzamide, and physiologically acceptable salts or solvates thereof.
35. The compound of claim 32, wherein the compound is selected from the group consisting of N-(7-oxa-nonyl)-1,5,6-trideoxy-1,5-imino-D-galactitol (N-7-oxa-nonyl MeDGJ), N-(7-oxa-nonyl)-1,5-dideoxy-1,5-imino-D-galactitol (N-7-oxa-nonyl DGJ), and physiologically acceptable salts or solvates thereof.
36. A pharmaceutical composition comprising a nitrogen-containing virus-inhibiting compound and a pharmaceutically acceptable carrier, wherein the nitrogen-containing virus inhibiting compound includes an N-C₈-C₁₆ alkyl group.
37. A method of manufacturing a pharmaceutical composition comprising combining a nitrogen-containing virus-inhibiting compound with a pharmaceutically acceptable carrier, wherein the nitrogen-containing virus inhibiting compound includes an N-C₈-C₁₆ alkyl group.

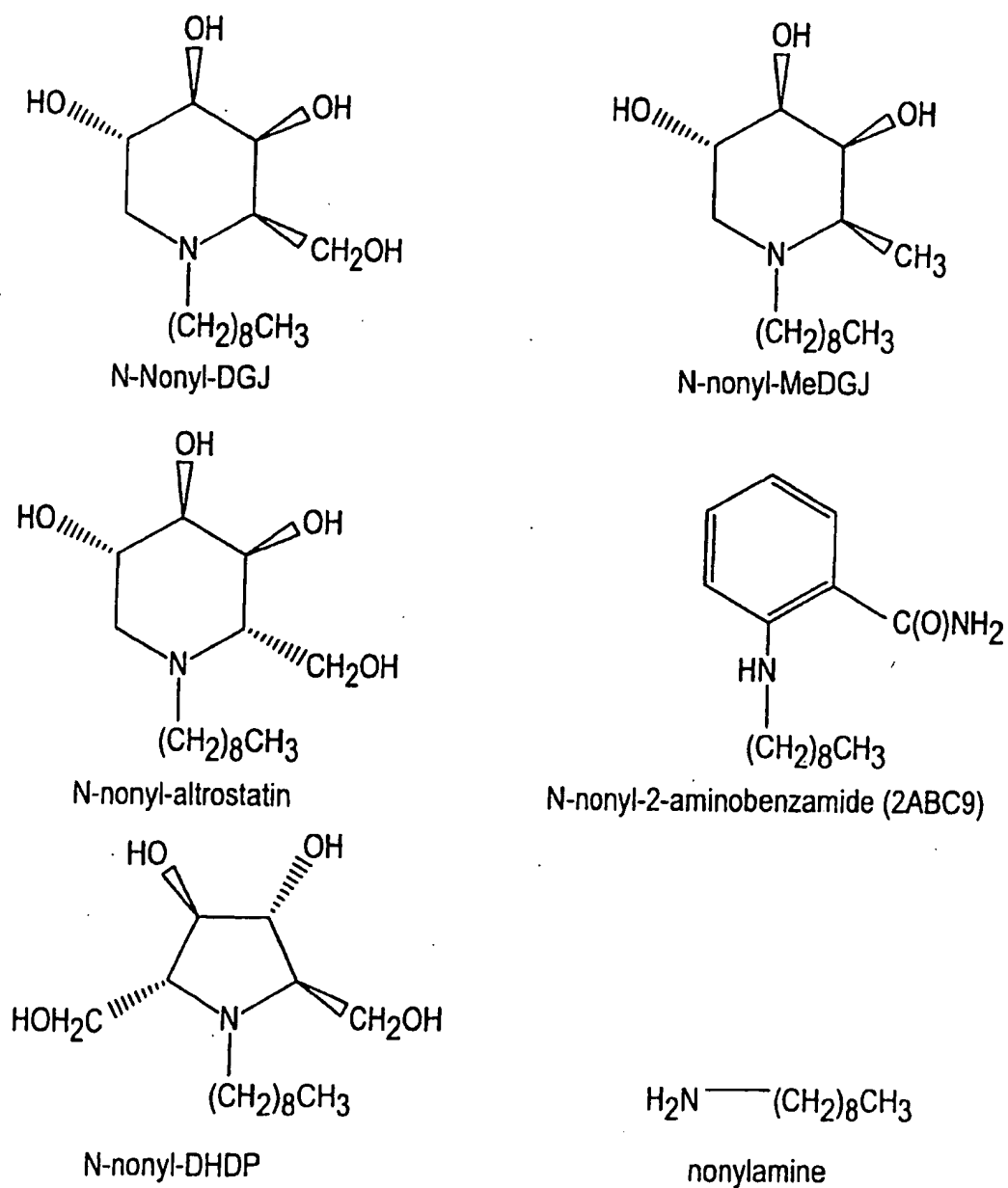


FIG. 1

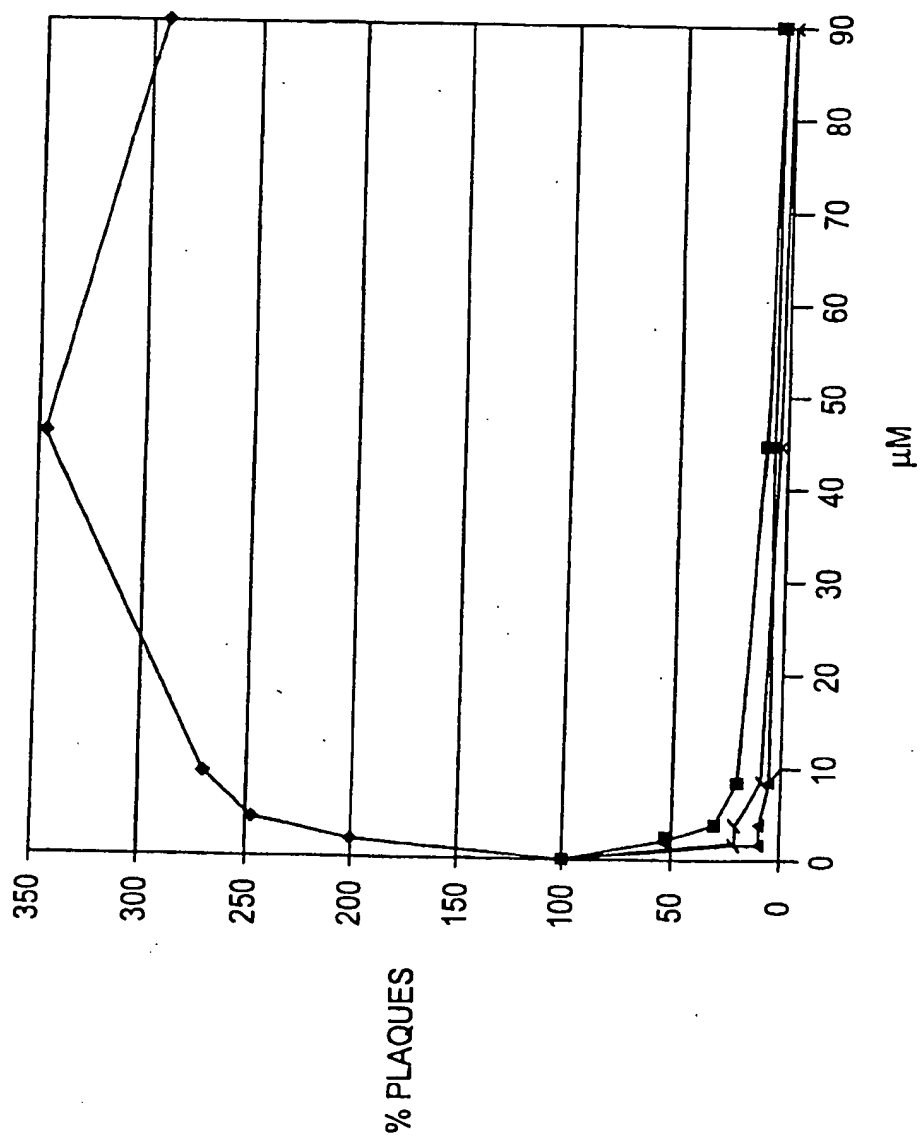


FIG. 2

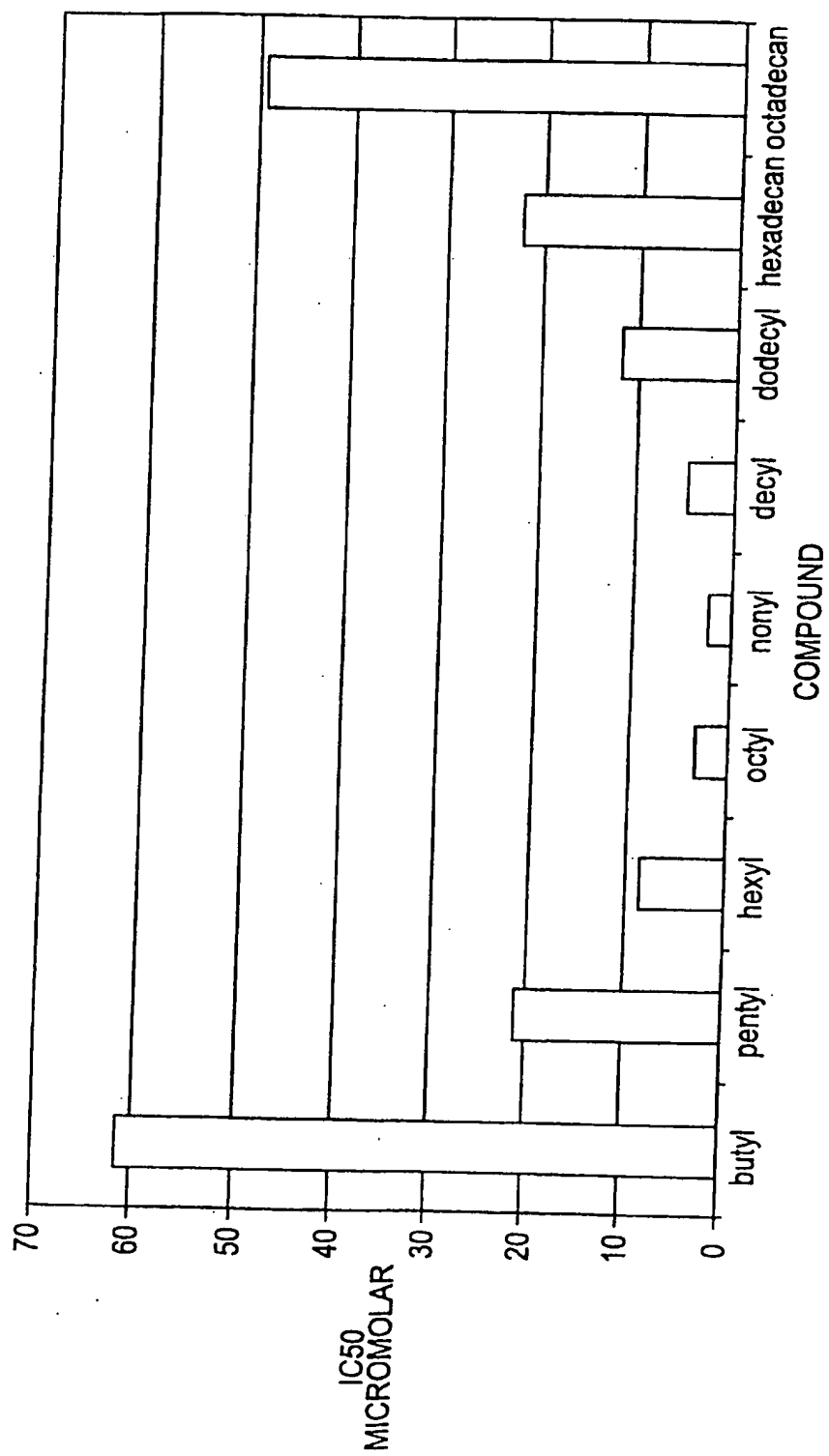


FIG. 3

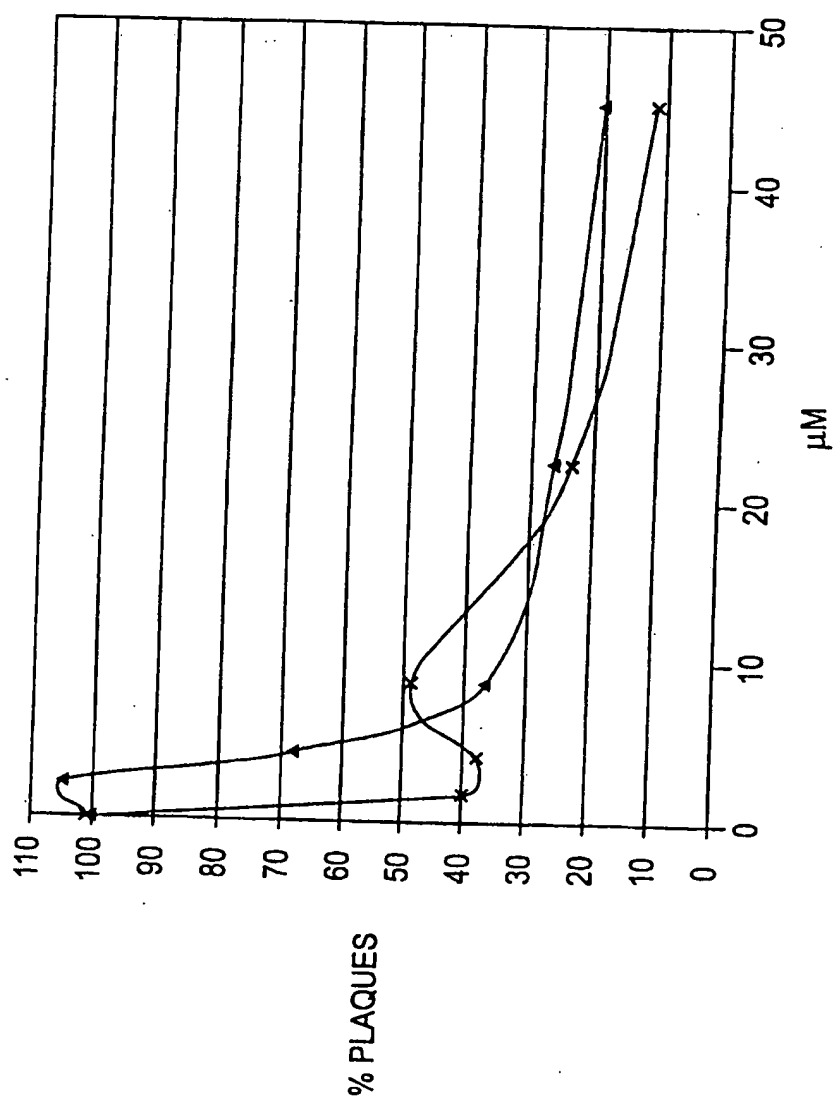


FIG. 4

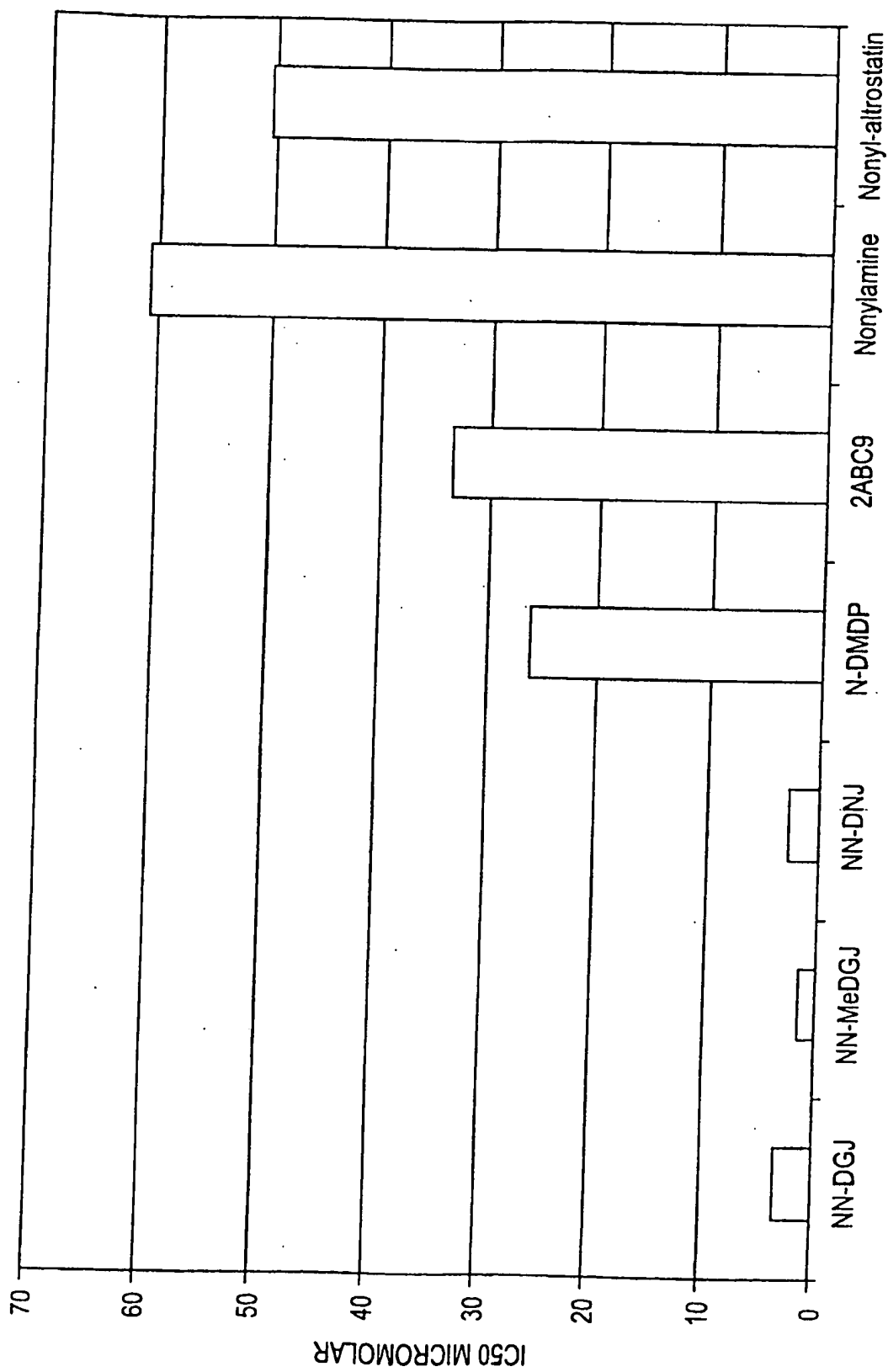


FIG. 5

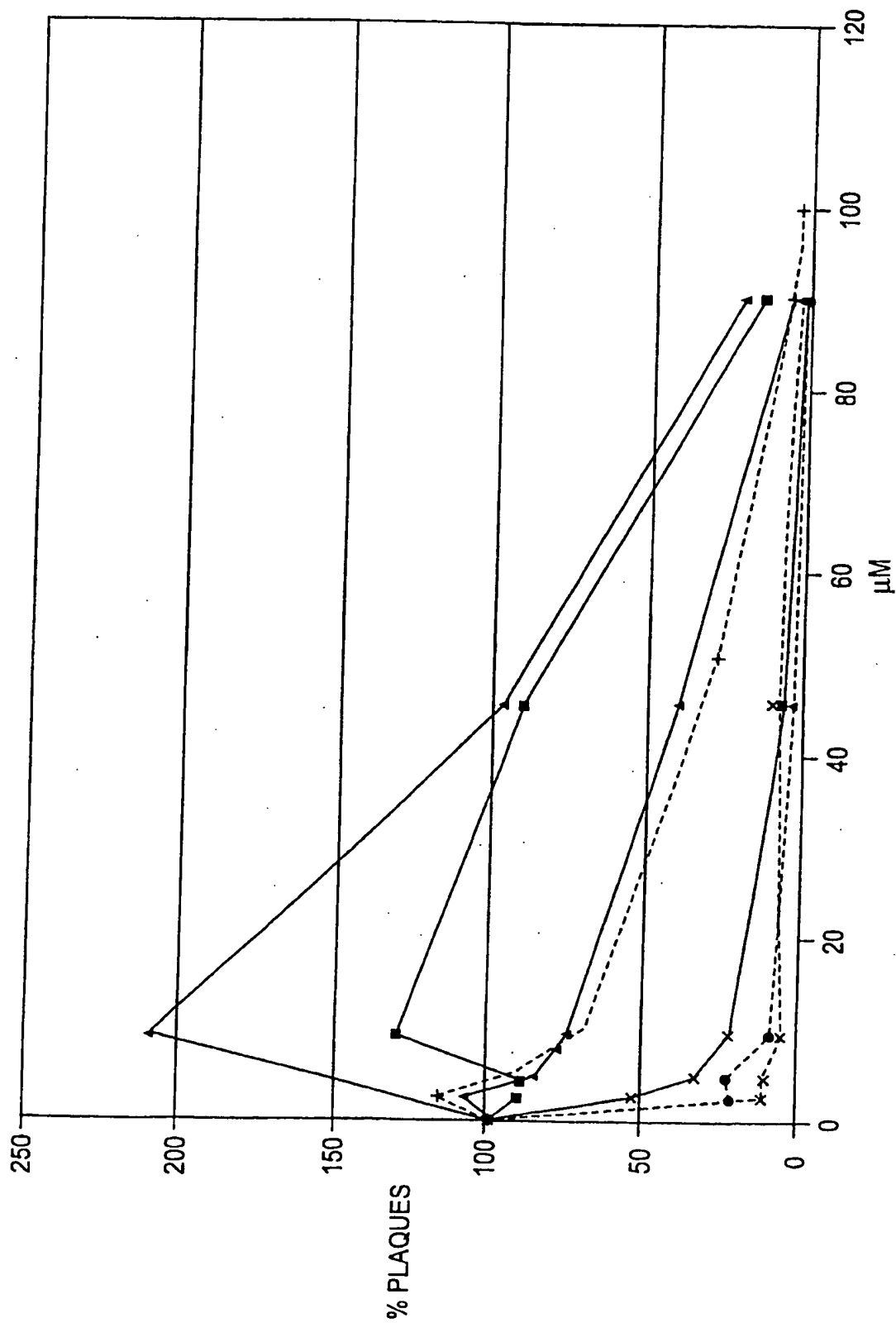


FIG. 6

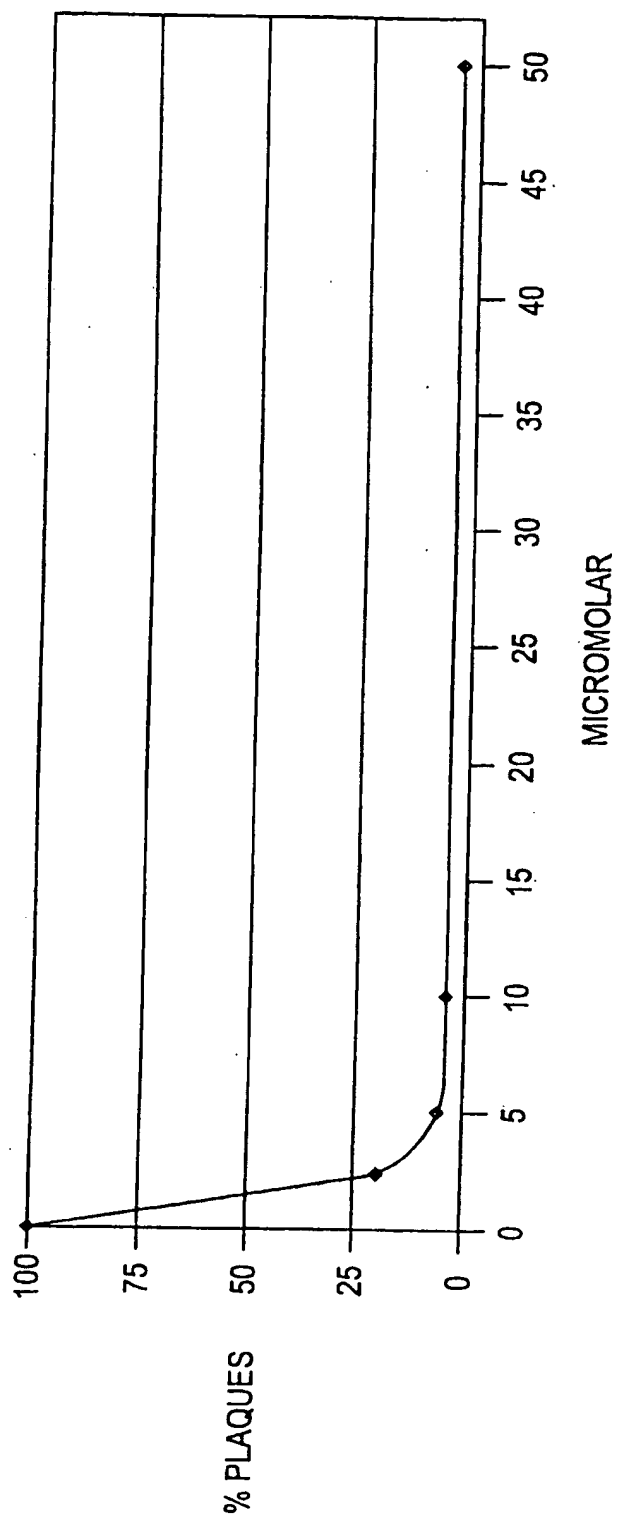


FIG. 7

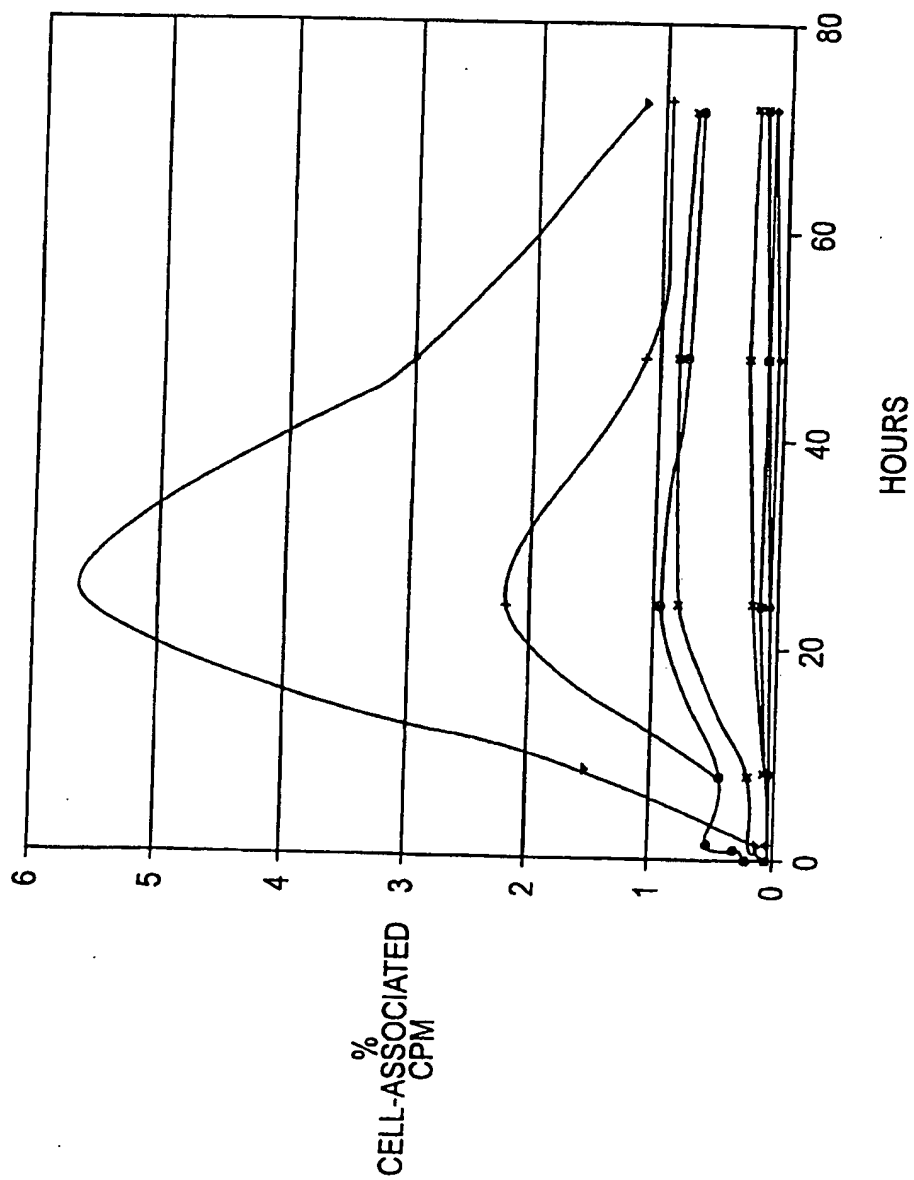


FIG. 8